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# Bioproduction and Characterization of a pH Responsive Self-Assembling Peptide

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**ABSTRACT:** Peptide $P_{11}$-4 (QQRFEWEFEQQ) was designed to self-assemble to form β-sheets and nematic gels in the pH range 5–7 at concentrations ≥12.6 mM in water. This self-assembly is reversibly controlled by adjusting the pH of the solvent. It can also self-assemble into gels in biological media. This together with its biocompatibility and biodegradability make $P_{11}$-4 an attractive building block for the fabrication of nanoscale materials with uses in, for example, tissue engineering. A limitation to large-scale production of such peptides is the high cost of solid phase chemical synthesis. We describe expression of peptide $P_{11}$-4 in the bacterium *Escherichia coli* from constructs carrying tandem repeats of the peptide coding sequence. The vector pET31b+ was used to express $P_{11}$-4 repeats fused to the ketosteroid isomerase protein which accumulates in easily recoverable inclusion bodies. Importantly, the use of auto-induction growth medium to enhance cell density and protein expression levels resulted in recovery of 2.5 g fusion protein/L culture in both shake flask and batch fermentation. Whole cell detergent lysis allowed recovery of inclusion bodies largely composed of the fusion protein. Cyanogen bromide cleavage followed by reverse phase HPLC allowed purification of the recombinant peptide with a C-terminal homoserine lactone ($rP_{11}$-4(hsl)). This recombinant peptide formed pH dependent hydrogels, displayed β-structure measured by circular dichroism and fibril formation observed by transmission electron microscopy.

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**KEYWORDS:** self-assembling peptide; nanostructured material; recombinant peptide

## Introduction

Peptide $P_{11}$-4 (QQRFEWEFEQQ) was rationally designed to form β-sheets and nematic gels in the pH range 5–7 at a concentration of 12.6 mM in water (Aggeli et al., 2003; Carrick et al., 2005; Davies et al., 2006; Kayser et al., 2004). This peptide when chemically synthesized has $CH_3CO$– and –$NH_2$ blocking groups at the N- and C-terminal ends respectively, thereby neutralizing the positive and negative terminal charges associated with a naturally occurring peptide sequence; we refer to this chemical form as $cP_{11}$-4. By adjusting the pH and concentration of an aqueous solution of this peptide, a reversible reaction between nematic gel and the isotropic fluid phase can occur. This responsive behavior is governed by the choice of amino acid side chains within the peptide enabling the hierarchical assembly of β-sheets through chemical and structural complementarity. Extensive characterization of the properties of $cP_{11}$-4 has been reported by Carrick et al. (2007). Biocompatibility with mammalian cells in tissue culture and the inherent biodegradability of $P_{11}$-4 make it useful as a nanoscale self-assembling material with applications in tissue treatment (Bell et al., 2006; Firth et al., 2006) and bone/dental remineralization (Kirkham et al., 2007). A chemical approach to peptide synthesis is convenient for high throughput analysis of peptide functionality at the design and testing stage, as it is rapid and can be performed at small-scale in parallel. However, large scale chemical production of peptides and proteins is difficult, expensive (Latham, 1999), and generates toxic chemical waste products.

An early example of recombinant expression of tandem peptide repeats was reported by Kuliopulos and Walsh (1994). The fusion protein ketosteroid isomerase (KSI) from *Pseudomonas testosterone* (Kuliopulos et al., 1987) is a 125 amino acid protein found in inclusion bodies when expressed in *Escherichia coli*. Targeting peptide fusions in this way allows protection from proteolytic digestion, prevents adverse effects on cells and facilitates simple recovery. The use of the restriction enzyme *Alw*NI for

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cloning the peptide repeats allowed the introduction of a methionine residue between the KSI protein and each peptide repeat and the C-terminal His-tag. Following purification, the fusion protein was subjected to cyanogen bromide (CNBr) cleavage resulting in the release of the peptide unimers each with a C-terminal homoserine lactone (hsl). Following HPLC purification a yield of 50 – 55 mg/L of pure peptide was obtained. More recently this system has been used for the production of an antimicrobial peptide, dermcidin, a 47 amino acid peptide that is active against the host organism *E. coli*. Cipakova et al. (2006) recovered 2.3 mg of active dermcidin. The ability to recover such a toxic peptide from this bacterial host confirms that inclusion bodies provide an effective location for protecting host cells against deleterious peptide products.

With regard to biological expression of self-assembling peptides only a few examples have been reported. Using the KSI fusion approach Sharpe et al. (2005) generated tandem repeats of the $A\beta_{11-26}$ peptide region. Reed et al. (2006) used the Gram negative bacterium *Ralstonia eutropha* to express the self-assembling peptide, RAD16, with a repeat unit of N-RADARADARADARADAE-C as a cellulose binding domain fusion. van Hell et al. (2007) expressed self-assembling vesicle-forming peptides using a pET-SUMO system. Most recently, Hartmann et al. (2008) also used the KSI-system to express tandem repeats of the peptide $P_{11}$-2, an 11 amino acid peptide (QQRFQWQFEQQ) (Aggeli et al., 2001b) related to $P_{11}$-4 (Aggeli et al., 1997).

In the present study, we have exploited the pET31b+ expression system (Novagen; Fig. 1) based on the KSI fusion (Kuliopulos and Walsh, 1994) to clone repeats of the coding region for the β-structured recombinant peptide termed $rP_{11}$-4, with intervening Met residues for cyanogen bromide cleavage. We have optimized aspects of the expression system including selection of a suitable host strain and the use of the auto-induction system of Studier (2005). The recombinant $P_{11}$-4 unimers display self-assembly forming a pH responsive hydrogel, adopting a β-structure as shown by circular dichroism (CD) and assembling into fibrils revealed by transmission electron microscopy (TEM).

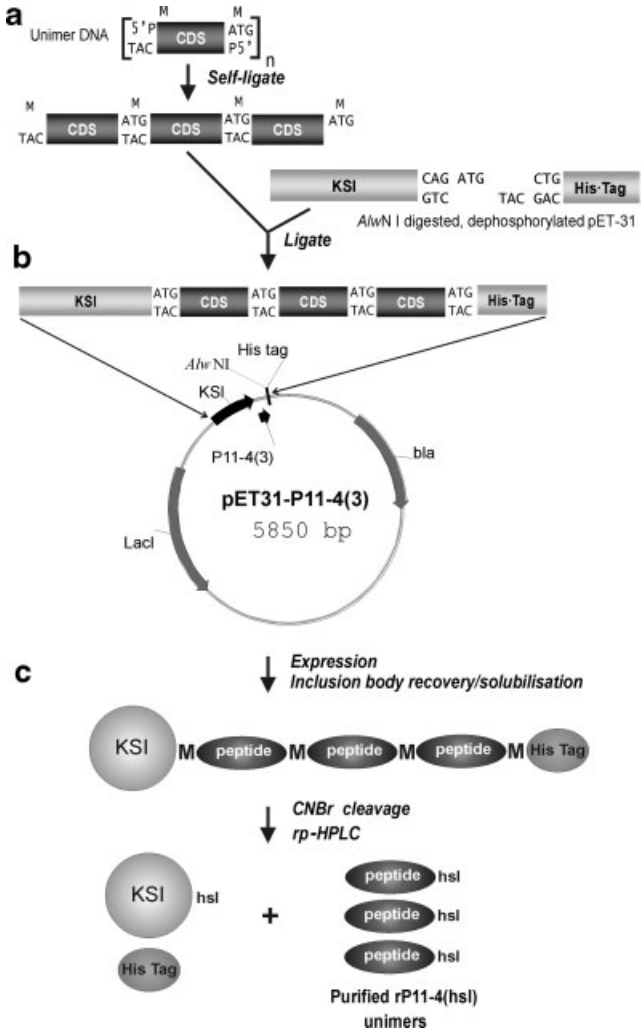


**Figure 1.** Cloning and expression strategy for $rP_{11}$-4 peptide repeats. **a**: Oligonucleotide coding sequences (CDS) were designed to introduce intervening methionine codons (ATG) when annealed and cloned. Tandem repeats were prepared by self-ligation of the cohesive ends and (**b**) ligated into the pET31b+, vector at the *Alw*NI site in-frame with the KSI coding region. **c**: Following protein expression and recovery and solubilization of inclusion bodies the fusion protein is susceptible to CNBr cleavage at the methionine (M) residues to generate a mixture of KSI, His-tag and $rP_{11}$-4(hsl) unimers with recovery of $rP_{11}$-4(hsl) by reverse phase HPLC.

## Materials and Methods

### Bacterial Strains, Plasmids, and Cell Culture Media

*E. coli* XL1 Blue (Stratagene, La Jolla, CA.) was used for routine cloning. *E. coli* expression strains used were: BLR (DE3) pLysS (Novagen; Merck Chemicals, Nottingham, UK), BL21 (DE3) Star (Invitrogen, Carlsbad, CA), Origami B (DE3) (Invitrogen), BL21 (DE3) Gold (Stratagene). The Expression Vector pET31b+ was from Invitrogen.

The growth media Luria Burtani broth (LB), Terrific Broth (TB), 2YT, SOC, and LB agar were prepared according to Sambrook et al. (1989). Auto-induction media were prepared as described by Studier (2005). Carbenicillin was added to media at a final concentration of 100 μg/mL. Cultures were grown in an orbital incubator at 37°C with shaking at 250 rpm unless otherwise stated. For transformations, cells were made chemically competent using the rubidium chloride method and were transformed with plasmid DNA (Sambrook et al., 1989).

### Design of $rP_{11}$-4 Encoding DNA Sequence

Two complementary oligonucleotides encoding $rP_{11}$-4 were designed, and synthesized with cohesive ends corresponding to Met codons (underlined) and were phosphorylated at the 5′-termini

```
        Q   Q   R   F   E   W   E   F   E   Q   Q   M
  5'P-cag caa cgc ttc gaa tgg gaa ttt gag caa cag atg-3'
3'-tac gtc gtt gcg aag ctt acc ctt aaa ctc gtt gtc-P5'
```

The oligonucleotides (100 pmol/μL in TE buffer; 10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) were combined in equimolar amounts (2,000 pmol) with 20 μL of 10× annealing buffer (400 mM Tris–HCl, pH 8.0, 100 mM $MgCl_2$, 500 mM NaC) and the final volume adjusted to 200 μL with water. The mixture was heated to 99°C for 10 min in a thermal cycler and allowed to cool to 30°C over a 15 min period. To purify the annealed product, 0.1 vol 3 M sodium acetate, pH 6.0, and 2 vol absolute ethanol were added, mixed and incubated at −20°C for at least 1 h. The sample was centrifuged for 5 min at 13,000*g*, the pellet washed with 70% ethanol, air dried then resuspended in TE buffer at 100 pmol/μL.

## Cloning of $rP_{11}$-4 Repeats Into pET31b

Tandem repeat sequences encoding $rP_{11}$-4 peptides were generated (Fig. 1a) by a modified version of the Novagen protocol for cloning into the pET31b+ expression vector (Fig. 1b). The ligation reaction contained 5 μL phosphorylated, annealed oligos (500 pmol), 2.5 μL 10X ligase buffer (New England Biolabs (NEB), Hitchin, UK), 16.5 μL double distilled water and 1 μL T4 DNA ligase (NEB) and was incubated at 16°C overnight. Following electrophoresis through a 3% low melting point agarose (Sigma, Gillingham, UK) containing 0.5 μg/mL ethidium bromide for 3 h at 4°C, ligated multimer DNA fragments were excised and purified using a QIAquick gel extraction kit (Qiagen, Crawley, UK).

Parallel ligation reactions were set up containing 6 μL (50 ng) of DNA containing 1–6 repeats, 2 μL (100 ng) *Alw*NI digested and dephosphorylated pET31b+ DNA, 1 μL 10X ligase buffer and 1 μL T4 DNA ligase (NEB) and incubated at 16°C overnight. A 5 μL aliquot of each reaction was used to transform competent *E. coli* XL1-Blue cells and colonies were screened by colony PCR and selected clones analyzed by DNA sequencing to confirm the repeat number and integrity.

## Colony PCR Screening

Colonies were transferred to 50 μL sterile $H_2O$, boiled at 100°C for 5 min, centrifuged at 13,000*g* for 1 min and 10 μL of supernatant used as template in a PCR reaction. The primers (100 pmol each); KSI (GGCAAGGTGGTGAGCATC) and T7 terminator (GCTACTTATTGCTCAGCGG) (MWG Biotech, Ebersberg Germany), were used in a 50 μL reaction volume comprising 1× KOD buffer, 0.2 mM dNTPs, 1 mM $MgSO_4$ and 1 U KOD Hot Start DNA polymerase (Novagen). The PCR was 1 cycle at 94°C for 1 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final extension 72°C for 1 min PCR products were analyzed on a 1.5% agarose gel. Constructs will be referred to by the following nomenclature KSI/$rP_{11}$-4(*N*)/His where *N* represents the number of peptide repeat units.

## DNA Sequencing

Clones were sequenced using the T7 promoter (TAATCGACTCACTATAGGG) and T7 terminator primers and products analyzed on an ABI 3100XL Capillary Sequencer (DNA Analysis Facility, Biological Sciences, University of Leeds).

## Protein Expression

### *Isopropyl-β-D-Thiogalactopyranoside (IPTG) Induction*

Starter cultures (1.5 mL) from single colonies were incubated with shaking at 37°C overnight, then 1 mL was added to 50 mL LB medium in a 250 mL conical flask and grown to $OD_{600}$ ~0.6 (2–3 h). Protein expression was induced by addition of IPTG (Sigma) to a final concentration of 1 mM and incubation was continued at 37°C for 16 h. For 500 mL trials a starter culture of 25 mL was grown as above and a 12.5 mL aliquot added to 500 mL media in a 2 L glass conical flask grown and induced as above.

### *Auto-Induction*

The protocols developed by Studier (2005) were used. A single colony was inoculated into a 5 mL overnight starter culture in LB medium. A 200 μL aliquot was then used to inoculate 400 mL auto-induction medium in a baffled conical flask which was incubated at 25°C with shaking. For time course expression experiments, 1 mL aliquots were taken between 16 and 60 h after inoculation. The components of various auto-induction media are given in Table I.

### *Fermentation*

A large scale expression trial was carried out in a 30 L Applikon ADI1075 bioreactor (Astbury Centre for Structural Molecular Biology, University of Leeds). An overnight starter culture (10 mL) carrying the pET31b_KSI/$rP_{11}$-4(3)/His expression plasmid was used to inoculate 300 mL of LB medium. This culture was grown for ~8 h (final $OD_{595}$ of 1.37) and used to inoculate a 30 L culture using TB auto-induction medium, scaled up from the 400 mL shake flask trial. Temperature (25°C), agitation (355 rpm; equivalent to 220 rpm in a Sanyo orbital incubator) and air flow (25%) were controlled by an AD1010 controller and logged using BioXpert software. As the head space volume in the vessel was only 25% compared to 80% in 2 L shake flasks, the constant flow of air to the fermenter was set at 15 L/min (0.5 vvm) to provide oxygen to the growing cells. Antifoam (Dow Corning, Coventry, UK) was added at 50% antifoam/50% water as required and pH was not controlled. Culture samples (15 mL) were taken at intervals until the final harvest time at 42 h after inoculation.

**Table I.** Composition of auto-induction media (adapted from Studier, 2005).

| Medium components | Growth Medium | | | | |
|---|---|---|---|---|---|
| | LB+ | TB+ | 2YT+ | 8ZY+ | 8ZY-2L+ |
| Tryptone (g/L) [Z] | 10 | 12 | 16 | 80 | 80 |
| Yeast extract (g/L) [Y] | 5 | 24 | 10 | 40 | 40 |
| NaCl (g/L) | 10 | | 5 | | |
| NPCS (mM) | | | | | |
| $Na_2HPO_4$ | 25 | 97 | 25 | 25 | 25 |
| $KH_2PO_4$ | 25 | 42 | 25 | 25 | 25 |
| $NH_4Cl$ | 50 | 50 | 50 | 50 | 50 |
| $Na_2SO_4$ | 5 | 5 | 5 | 5 | 5 |
| $MgSO_4$ (mM) | 2 | 2 | 2 | 2 | 2 |
| Trace metals | 0.2× | 0.2× | 0.2× | 0.2× | 0.2× |
| 5,052 (%) | | | | | |
| Glycerol | 0.5 | 0.9 | 0.5 | 0.5 | 1.0 |
| Glucose | 0.05 | 0.05 | 0.05 | 0.05 | 0.1 |
| Lactose | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

## Preparation of Total, Soluble and Insoluble Cell Proteins

Cells were harvested at 8,000*g* in a fixed angle rotor. OmniCleave™ endonuclease (Epicentre; Cambio, Cambridge, UK; 1 μL) and chicken egg white lysozyme (Sigma; 1 mg/mL) was added per 20 mL lysis buffer (50 mM HEPES (pH 8.0), 25% (w/v) sucrose, 5 mM $MgCl_2$ and 1% (v/v) Triton X-100) immediately prior to use. Lysis buffer (20 mL) was added to the cell pellet from a 400 mL culture (200 μL for 1 mL pellets), with vigorous agitation at 4°C to resuspend the pellet and produce a homogenous lysate. Samples were removed as total cell protein fractions. The lysate was then centrifuged at 22,000*g* for 30 min to pellet the insoluble material. Aliquots of supernatant, representing the soluble protein fraction were reserved for analysis. The pellet was washed in 0.1X lysis buffer and centrifuged at 10,000*g* for 10 min. Routinely four washes were performed to give the inclusion body (insoluble) fraction.

## SDS–PAGE Analysis of Protein Expression and Densitometry Analysis

Protein samples were heated at 95°C for 5 min in SDS/β-mercaptoethanol loading buffer before analysis on 4–12% NuPAGE® Novex Bis–Tris precast gels (Invitrogen, Paisley, UK) at 190 V for 40 min. Protein bands were visualized using Coomassie brilliant blue and protein concentrations estimated by densitometry using Aida Image Analyser software. The intensities of the bands were compared with those of known concentrations of low molecular weight standards (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK).

## Protein Concentration by UV Spectroscopy

Insoluble pellets were resuspended in 6 M guanidium–HCl or 8 M urea and the absorbance read at 280 nm against control solutions. Concentrations were estimated according to the Beer–Lambert Law ($A = \varepsilon cl$) using the theoretical extinction coefficients shown in Table II and determined using ProtParam (http://ca.expasy.org/cgi-bin/protparam).

## Western Blot Analysis of Protein Expression

SDS gels were blotted onto Protran nitrocellulose transfer membrane (Sigma) using an electrophoretic transfer apparatus (Invitrogen, UK) for 1 h at 30 V. Non-specific binding sites were blocked in 30 mL of blocking buffer (5% (w/v) dried milk [Marvel] in transfer buffer) overnight at 4°C or for 1 h at room temperature with gentle shaking. The membrane was then washed three times with 30 mL 1 × PBS/0.1% Tween for 10 min with shaking. Following incubation with HisProbe-HRP (Pierce, Rockford, IL) at 1:3,000 dilution of a 4 mg/mL stock for 1 h at room temperature with agitation, the membrane was washed three times as before. ECL detection (Amersham) was performed according to the manufacturer's instructions and the membrane exposed to X-ray film (Kodak MXB) for up to 1 min then developed.

## Protein Purification by Immobilized Metal Affinity Chromatography (IMAC)

Inclusion body pellets containing KSI/$rP_{11}$-4(3)/His were resuspended in 125 mL binding buffer (20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 1 mM 2-mercaptoethanol, pH 8). The solution was stirred for 30–60 min at room temperature and centrifuged at 13,000*g* for 15 min at 4°C and the supernatant passed through a 0.45 μm filter to remove any insoluble material. A 5 mL HiTrap chelating column (GE Healthcare UK Ltd.) was used to purify KSI fusion proteins according to the manufacturer's instructions using an imidazole gradient of 0–500 mM. The peak fractions were located by $A_{280}$ readings and 10 μL samples were analyzed by SDS–PAGE.

**Table II.** Theoretical extinction coefficients (at 280 nm) for KSI/$rP_{11}$-4(N)/His tandem repeat proteins and for recombinant $rP_{11}$-4(hsl) with a C-terminal homoserine lactone.

| Protein/peptide | Extinction coefficient (theoretical) ($M^{-1}\,cm^{-1}$) |
|---|---|
| KSI/$P_{11}$-4(1)/His | 8480 |
| KSI/$P_{11}$-4(2)/His | 13980 |
| KSI/$P_{11}$-4(3)/His | 19480 |
| KSI/$P_{11}$-4(4)/His | 24980 |
| KSI/$P_{11}$-4(5)/His | 30480 |
| KSI/$P_{11}$-4(6)/His | 35980 |
| $rP_{11}$-4(hsl) | 5500 |

### Cleavage of the KSI Fusion Protein by Cyanogen Bromide

Purified inclusion bodies of KSI/$rP_{11}$-4(3)/His were dialyzed against 5,000-fold excess water overnight and then resuspended to a concentration of 10 mg/mL in 80% formic acid. Cyanogen bromide (CNBr; Sigma) was dissolved in 80% formic acid and added to the protein sample at a 200 molar excess to methionine residues in a 2 mL volume. The vial was flushed and sealed with $N_2$ and incubated in the dark overnight with gentle agitation. The formic acid was removed by rotary evaporation until a gelatinous material formed which was then either resuspended in 8 M urea for SDS–PAGE analysis or resuspended and dialyzed against water pH 9.0 for purification of cleaved $rP_{11}$-4 unimers. The latter samples were centrifuged at 22,000*g* for 30 min to separate insoluble white precipitate from the supernatant. The supernatant was used for reverse phase HPLC purification. The $rP_{11}$-4 unimers generated by CNBr cleavage contain a C-terminal homoserine lactone and so we refer to this material as $rP_{11}$-4(hsl) to indicate the recombinant and modified nature of this peptide.

### Reverse-Phase HPLC of CNBr Cleaved Peptide

Reverse phase HPLC was performed on a Dionex Summit machine using Chromeleon software for peak analysis. The column was a C18 (octadecyl derivatized silica) used at a flow rate of 1 mL/min. Buffer A (5% acetonitrile/95% water) and buffer B (95% acetonitrile/5% water) were both adjusted to pH 9.0 with NaOH or $NH_4OH$ to favor the monomeric peptide state. The CNBr cleaved sample was dialyzed against 5,000-fold excess water overnight at room temperature and filtered (0.45 μm) prior to loading. The column was equilibrated in buffer A, the sample was loaded and the column was then re-equilibrated with ~5 mL of 1% buffer B and a gradient elution of 1–80% buffer B was performed over 15 mL. Eluate was analyzed by measuring absorbance at 280 and 215 nm. The peaks were identified by mass spectrometry and $rP_{11}$-4(hsl) unimer was lyophilized for storage.

### Mass Spectrometry (MS)

Peptide or protein samples were prepared by dissolving dried powders in 20 μL of methanol and 1 μL of 100% formic acid for analysis on a Platform II Electrospray Ionization MS instrument for molecular weight determinations. Samples for sequence determination were analyzed by tandem mass spectrometry (MS/MS) (MS Facility, Astbury Centre, University of Leeds).

### Formation of Peptide Gel

Lyophilized $rP_{11}$-4(hsl) unimer was resuspended to 6.7 mM in water pH 2.0 to instantaneously form a self-supporting hydrogel. To reverse this gel state NaOH was added until an isotopic fluid was observed.

### Transmission Electron Microscopy (TEM)

Cells from auto-induction expression (40 h) were treated by fixation with glutaraldehyde then with osmium tetroxide before setting in resin allowing thin (50 – 100 nm) sectioning of the specimen and negative staining with uranyl acetate. For analysis of peptide $rP_{11}$-4(hsl) fibrils, solutions were prepared by dilution of a peptide hydrogel sample to ~125 μM in water. Glow-discharged and carbon coated TEM copper grids (hexagonal mesh size 400 [Agar]) were covered with diluted $rP_{11}$-4(hsl) solutions and allowed to adsorb for 1 min and were then negatively stained with uranyl acetate at 2% (w/v) in water. Air dried grids were analyzed using a Philips CM10 TEM or a Jeol 1200EX TEM operating at 80 or 100 kV accelerating voltage.

### Analysis of Peptide Secondary Structure by Circular Dichroism (CD) Spectroscopy

$rP_{11}$-4(hsl) samples for CD analysis were prepared by dilution of a hydrogel at 10 mg/mL in water (pH 2.0). Samples were sonicated for 15 min and then allowed to equilibrate at room temperature (25°C) for 3 days before CD analysis. Aliquots of $rP_{11}$-4(hsl) solutions were transferred to 0.1 mm (Hellma) quartz cuvettes and spectra recorded on a Jasco J-715 spectropolarimeter at 20°C in the far ultraviolet region ($\lambda = 190$–240 nm). Spectra were an average of five scans with a step resolution of 0.5 nm, scan speed 50 nm/min, response time 1 s, and sensitivity 50 millidegrees and baseline solvent (water) subtracted. The sample concentrations were checked by measuring absorbance at 280 nm after CD analysis.

## Results and Discussion

### Cloning and Expression Strategy

The scheme in Figure 1 illustrates the cloning and expression strategy adopted. The oligonucleotides corresponding to the complementary strands of the coding sequence for the self-assembling peptide $P_{11}$-4 (Aggeli et al., 2003), were designed with an overhanging Met codon sequence (ATG) and were phosphorylated at their 5′-termini. They were annealed and self-ligated and DNA fragments comprising 1–6 tandem repeats were recovered from an agarose gel and cloned into the *Alw*NI site of the pET31b+ expression vector. This vector contains the KSI gene from *P. testosterone* (Kuliopulos et al., 1987) that accumulates in inclusion bodies in *E. coli* cells (Kuliopulos and Walsh, 1994). A hexa-histidine tag (His-tag) at the C-terminus allows purification by IMAC. The Met residues can be cleaved by CNBr (Gross

and Morell, 1974; Kuliopulos and Walsh, 1994) to release unimer peptides.

The presence of correct inserts was verified by colony PCR and representative clones were subjected to DNA sequence analysis to verify the correct sequence and orientation of peptide repeats.

## Expression of KSI Fusion Constructs in *E. coli* by IPTG Induction

Constructs were initially transformed into the *E. coli* strain BLR (DE3) pLysS (Novagen) a *recA*$^-$ derivative of BL21 suitable for stabilizing plasmids containing repetitive sequences (Studier, 1991). Cultures in 50 mL LB were induced by IPTG addition and the insoluble fractions, adjusted for culture density to ensure equivalent loading, were analyzed by SDS–PAGE. Fusion protein of the expected size was seen in induced samples containing 1–6 repeats of the $rP_{11}$-4 coding sequence compared with the absence of corresponding protein in the un-induced control (Fig. 2a). The identity of the proteins was confirmed by western blot analysis using HisProbe-HRP detection of the His tag (Fig. 2b). Protein bands, migrating to give the same pattern but at a higher molecular mass, are dimers as noted previously for peptide fusions (Zhong et al., 2006).

These results demonstrate expression of the fusion proteins, although there is evidence, reinforced when 500 mL IPTG induced cultures were tested (data not shown), that with increased repeat length, protein expression levels decreased. This effect was most pronounced for the 4–6 repeats. As we were interested in enhancing the yield of fusion protein and peptide we tested levels of expression achievable with auto-induction media.

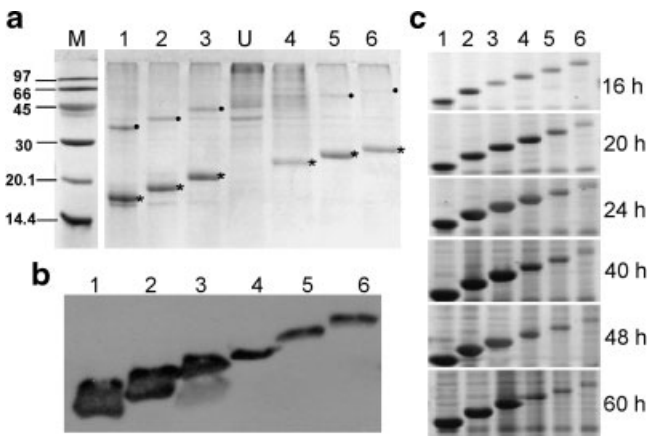


**Figure 2.** Expression of KSI/$rP_{11}$-4(1-6)/His constructs. **a**: SDS–PAGE analysis of small-scale (50 mL) IPTG-induced expression trial of KSI fusion constructs carrying tandem repeats (1–6) of $rP_{11}$-4. Sample U is a KSI/$rP_{11}$-4(4)/His un-induced control. Equivalent quantities of protein were loaded. Target fusion protein (*) and probable dimers (•) are indicated. M, size markers indicated in kDa. **b**: Western blot of SDS–PAGE of IPTG induced KSI/$rP_{11}$-4(1–6)/His constructs using HisProbe-HRP. **c**: SDS–PAGE analysis of auto-induction in TB media of KSI/$rP_{11}$-4(1–6)/His constructs sampled at the indicated times (hours post inoculation). Equal volumes of insoluble cell protein samples were loaded to compare the levels of expression.

## Expression of KSI Fusion Constructs by Auto-Induction

Studier (2005) developed media and additives which allow cells to grow to very high cell density ($OD_{600} > 20$) on glucose, before switching to glycerol and lactose upon glucose depletion, with consequent formation of allolactose the natural inducer of the *lac* operator/promoter region. The KSI/$P_{11}$-4(3)/His construct was selected for initial optimization experiments. A range of BL21 expression hosts were tested and it was noted that the use of BLR was unnecessary for maintaining the repeat-containing plasmids. The strain BL21(DE3) Star (Stratagene), which carries a mutated *rne* gene (*rne131*) encoding a truncated RNaseE resulting in increased mRNA stability, showed the highest level of fusion protein expression and was used in subsequent auto-induction experiments. Different auto-induction growth media were tested (Table I). Total insoluble cell material was analyzed by SDS–PAGE and target protein concentration was estimated by densitometry. By 44 h, the longest time point tested, the TB cultures had reached an $OD_{600}$ of 26, compared with values of 22 and 20 for LB and 2TY, respectively, and growth had reached a plateau. For the richer media 8ZY and 8ZY2L the cultures had only reached an $OD_{600}$ of 14 by 44 h but had not reached stationary phase. For subsequent experiments TB medium has been used since it provided high yields within a reasonable timeframe. However, it seems likely that enhanced levels of protein would be achievable by allowing the richer medium cultures to grow for longer periods of time.

Constructs carrying 1–6 repeats of $rP_{11}$-4 were analyzed for peptide yield by growth under auto-induction conditions in TB at 25°C with samples taken at 16, 20, 24, 40, 48, and 60 h after inoculation. Insoluble proteins, isolated from equivalent cell mass samples, were analyzed by SDS–PAGE. Figure 2c demonstrates that good yields of fusion proteins were achievable and reinforces the observation, from IPTG induction studies, that as the number of $rP_{11}$-4 repeats increases, the level of fusion protein detected decreases. Clones expressing proteins with 1–3 repeats allowed recovery of high levels of protein at 60 h. As shown in Table III, the KSI fusion protein (125 aa) and His tag (9 aa) together account for the majority of the expression product. Quantitative analysis of fusion protein yields from each construct and calculation of the theoretical proportion of $rP_{11}$-4 peptide reveal that construct KSI/$rP_{11}$-4(3)/His, although not producing as much fusion protein as the 1 or 2 repeat constructs, would actually provide the highest theoretical yield of $rP_{11}$-4 unimer (Table III).

There have been varied reports of success from attempts to increase peptide yield by cloning peptide coding sequences as tandem repeats. Kuliopulos and Walsh

**Table III.** Proportion of each fusion protein that comprises $rP_{11}$-4 peptide.

| Construct | Number of amino acids | | %$rP_{11}$-4 in fusion protein | Maximum yield of $rP_{11}$-4(hsl) at 44 h cell growth (g/L) from estimation of fusion protein levels |
|---|---|---|---|---|
| | In fusion protein | In $rP_{11}$-4 repeats | | |
| KSI/$rP_{11}$-4(1)/His | 146 | 12 | 8 | 0.23 |
| KSI/$rP_{11}$-4(2)/His | 158 | 24 | 15 | 0.30 |
| KSI/$rP_{11}$-4(3)/His | 170 | 36 | 21 | 0.53 |
| KSI/$rP_{11}$-4(4)/His | 182 | 48 | 26 | 0.31 |
| KSI/$rP_{11}$-4(5)/His | 194 | 60 | 31 | 0.21 |
| KSI/$rP_{11}$-4(6)/His | 206 | 72 | 35 | 0.09 |

(1994) described maximum yields from 3 repeats of a yeast α-mating factor fused to KSI with lower yields for 5 and 6 repeat constructs, as observed in the present study. Also with the KSI system (Sharpe et al., 2005) produced tandem repeats of the $A\beta_{11-26}$ peptide from the Alzheimer's beta amyloid protein. They found that peptide yields were greatest from a 3 repeat construct. Hartmann et al. (2008) have expressed tandem repeats of the peptide $P_{11}$-2, which is related to $P_{11}$-4, using the KSI system but found in this case that a single repeat gave the highest yield of peptide. There are also examples where increasing peptide tandem repeat number has improved potential yield. The antimicrobial peptide, buforin II, was fused to an acidic peptide in tandem repeats of 1, 2, 4, and 6 units and maximal yields were observed for the 6 buforin II repeats (Lee et al., 1998). Also, oligomeric metallothionein genes from *Neurospora crassa* were expressed as a fusion to a maltose-binding protein (MalE) and highest level expression was observed with 9-mer constructs. Thus it seems that the most appropriate number of repeats to include in a construct for a given peptide and expression system requires some empirical analysis.

## Examination of Intracellular Inclusion Bodies

TEM was used to examine auto-induced cells for the presence of electron dense inclusion bodies. Figure 3a shows a typical cell carrying the KSI/$rP_{11}$-4(3)/His construct under non-induced conditions and is characteristic of a normal *E. coli* cell with no inclusion bodies. By contrast Figure 3b shows a typical cell carrying the same construct under induced conditions and reveals electron dense regions at the poles of the cell, typical of protein-derived inclusion bodies. This partitioning into inclusion bodies of fusion proteins carrying self-assembling peptides is convenient as it allows the rapid isolation of partially purified material and the harsh denaturing conditions required for solubilization do not affect the integrity of the peptides as they have no inherent tertiary structure.

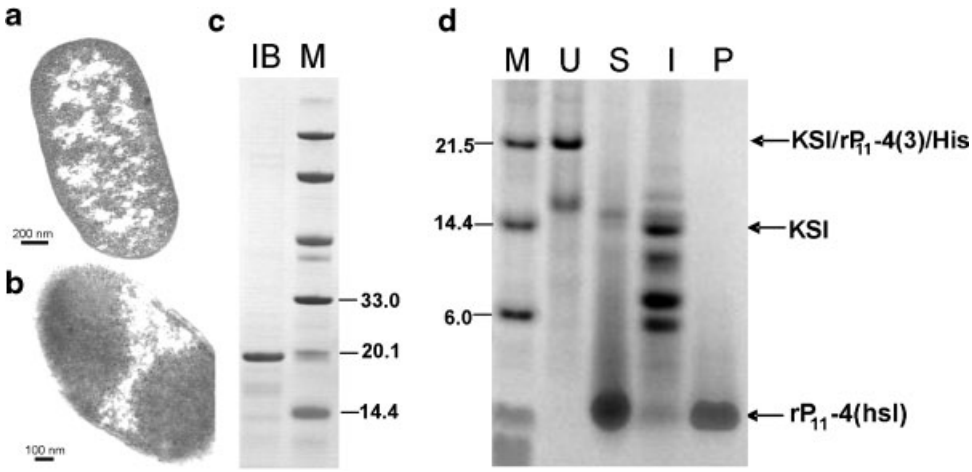


**Figure 3.** Inclusion body recovery and CNBr cleavage. TEM analyses of *E. coli* cells carrying a pET31/rP11-4(3) construct (**a**) un-induced and (**b**) induced and showing dense inclusion bodies. **c**: SDS–PAGE analysis of inclusion body sample of KSI/$rP_{11}$-4(3)/His; IB is a 0.1-fold dilution of a solubilized inclusion body sample, M is molecular weight markers with relevant sizes indicated in kDa. **d**: SDS–PAGE of purification of $rP_{11}$-4(hsl) showing KSI/$rP_{11}$-4(3)/His fusion protein before CNBr cleavage (U), CNBr cleavage products, soluble fraction (S) and insoluble fraction (I) and rp-HPLC purified $rP_{11}$-4(hsl) (P). The expected positions of species are labeled. M, size markers indicated (kDa).

### Cell lysis and Purification of KSI/rP$_{11}$-4(3)/His Inclusion Bodies

Cells from auto-induction of BL21 Star (DE3)/pET31-KSI/rP$_{11}$-4(3)/His were lyzed using a simple and rapid detergent lysis approach and inclusion bodies were recovered by centrifugation. The wash steps to remove contaminating material resulted in greater purity of target protein, and typically four washes were performed with negligible loss of fusion protein. Analysis of inclusion body preparations, by SDS–PAGE and densitometry, allowed an estimate of ~90% purity for the fusion protein (Fig. 3c). This level of purity was equivalent to that obtained by IMAC purification and so to simplify the processing steps it was decided not to use IMAC for further purification of KSI/rP$_{11}$-4(3)/His. These experiments resulted in very good levels of recovery of fusion protein of ~2.5 g/L which corresponds to the expression of ~0.5 g/L rP$_{11}$-4(hsl).

### Batch Fermenter Expression of KSI/rP$_{11}$-4(3)/His in *E. coli*

Scale-up expression of BL21 Star (DE3)/pET31-KSI/rP$_{11}$-4(3)/His was performed in a 30 L Applikon ADI1075 fermenter in TB at 25°C for 42 h. Product yield at 42 h was again 2.5 g/L, indicating that levels of rP$_{11}$-4 produced in this fermentation (0.53 g/L) are similar to those of the 400 mL cultures in 2 L shake flasks. Optimization of fermentation conditions, for example, through modifying oxygen control regimes, would be expected to provide enhanced protein yields for large-scale production systems.

### Cyanogen Bromide Cleavage of KSI/rP$_{11}$-4(3)/His

Inclusion bodies from samples of either shake flask or fermenter studies, purified as above, were dialyzed against water for 24 h and the dense white precipitate was collected and dissolved in a minimal volume of 80% formic acid to give >2 mg/mL protein suspension. Following incubation with CNBr the formic acid was removed by rotary evaporation. Upon resuspending the resulting gelatinous material in water a number of crystals formed. These were dissolved in 8 M urea for analysis by SDS–PAGE. The dissolved crystals contained KSI protein (13.4 kDa) while the supernatant contained predominantly rP$_{11}$-4(hsl) (1.6 kDa) along with His-tag peptide (data not shown). There were also some fragments larger than KSI, most probably the fusion protein, indicating that cleavage by CNBr was not 100% efficient.

### Purification of rP$_{11}$-4(hsl) by Reverse Phase High Performance Liquid Chromatography

The CNBr-cleaved products were resuspended in water and the pH adjusted to 9 by addition of NaOH to ensure rP$_{11}$-4(hsl) unimers were in solution. The sample was incubated for two days at room temperature to promote full conformational equilibration of the peptide, and was then applied to a C18 column. The elution profiles were monitored at both 215 and 280 nm. Since rP$_{11}$-4(hsl) contains a tryptophan residue it is detectable at 280 nm, whereas the histidine tag would not absorb at 280 nm. Three peaks were detected at 215 nm and 2 peaks at 280 nm and were analyzed by ESI/MS; the major peak which absorbed at both 215 and 280 nm had a molecular mass of $1638.07 \pm 0.14$ while the second 215 and 280 nm peak had a molecular mass of $1637.19 \pm 0.19$. This suggests that both peaks contained the homoserine-lactone form (rP$_{11}$-4(hsl); expected $M_r = 1637.7$) and not the homoserine form (rP$_{11}$-4(hs); expected $M_r = 1655.7$) with the 1 Da mass difference likely to reflect the protonation state of the peptide. The final peak which did not absorb at 280 nm had a mass of $1195.78 \pm 0.43$ identifying it as the hexa-histidine fragment (LLEHHHHHH, $M_r = 1196.3$). This rp-HPLC approach to purification is not ideal as recovery of peptide was relatively low with 90 mg recovered from a sample that should theoretically yield 500 mg. The SDS–PAGE in Figure 3d shows the uncleaved (lane 1) and cleaved fusion protein (lane 2, soluble; lane 3, insoluble) and the corresponding rp-HPLC purified rP$_{11}$-4(hsl) which we estimate to be >99% pure.

Although our strategy requires further optimization in terms of purification efficiency, we have achieved the highest levels of fusion protein and unimeric self-assembling peptide recovery, for similar length peptides, so far reported (Table IV). van Hell et al. (2007) reported the purification and acetylation of 30 mg of the vesicle forming peptide SA2 from 300 mg, although this would represent essentially 100% efficiency, as the fusion protein is reported as 13 kDa and the peptide as 1.18 kDa. The level of peptide recovery achieved for P$_{11}$-2, a similar type of peptide to P$_{11}$-4, at 41%

**Table IV.** Reported levels of production and purification of self-assembling peptides by recombinant approaches.

| Peptide | Fusion protein yield (mg/L) | Theoretical peptide yield (mg/L) | Peptide purified (mg/L) | Efficiency of peptide purification (%) | Refs. |
|---|---|---|---|---|---|
| A$\beta_{11-26}$ | 80 | 27 | 10 | 37 | Sharpe et al. (2005) |
| RADA | 433 | 116 | 10.1 | 8.7 | Reed et al. (2006) |
| SA2 | 300 | 30 | 30 | 100 | van Hell et al. (2007) |
| P$_{11}$-2 | 85 | 6.8 | 2.63 | 41.7 | Hartmann et al. (2008) |
| P$_{11}$-4 | 2,500 | 530 | 90 | 17 | This work |

represents a good yield, although as it was isolated from a single repeat construct and overall levels of fusion protein were modest (85 mg/L) the amount of peptide recovered was only 2.63 mg/L (Hartmann et al., 2008). If we could achieve a similar level of recovery of ~40% with $rP_{11}$-4(hsl) this would equate to of 217 mg of peptide. However, it should be noted that the sequence differences between $P_{11}$-2 and $P_{11}$-4 result in substantial differences in their self-assembling properties which is likely to affect their behavior during purification.

To simplify the recovery of $rP_{11}$-4(hsl) an obvious modification of the pET31b+ cloning vector would be removal of the C-terminal His-tag which is not used for purification, thereby preventing this contamination of the $rP_{11}$-4(hsl) fraction.

### Peptide Sequencing by Tandem Mass Spectroscopy

The $rP_{11}$-4(hsl) major peak from rp-HPLC was subjected to peptide sequencing by Time of Flight MS/MS which provided data for 8 amino acids giving the sequence R-F-E-W-E-F-E-Q which correspond to residues 3–10 of the $rP_{11}$-4(hsl) peptide. These results further verify that a recombinant form of $P_{11}$-4 has been produced and purified.

### Formation of a Self-Supporting $rP_{11}$-4(hsl) Peptide Hydrogel in Water

Chemically synthesized $cP_{11}$-4 forms self-supporting hydrogels at 6.3 mM (~10 mg/mL) at pH 2–3.2 in water (Aggeli et al., 2003). Purified $rP_{11}$-4(hsl) was resuspended to 11 mg/mL (6.7 mM) in water at pH 2 and a self supporting hydrogel was observed to form immediately. A sample of $rP_{11}$-4(hsl) at this concentration was adjusted to pH 10 and underwent an immediate transition to a fluid. Figure 4a shows vials containing the hydrogel (pH 2) and fluid (pH 10) $rP_{11}$-4(hsl) samples illustrating the self-supporting nature of the hydrogel. To further characterize the hydrogel properties electron microscopy and CD spectroscopy were performed.

### Characterization of Fibril/Fiber Formation of Pure $rP_{11}$-4(hsl)

A sample of the $rP_{11}$-4(hsl) hydrogel was diluted in water (pH 2) to approximately 125 μM and stained with 2% uranyl acetate on carbon-coated copper grids for TEM analysis. Images of typical fibrils are shown in Figure 4b. The morphologies of these fibrils are well ordered and semi-rigid. A left handed helical twist is observed resulting from the L-chirality of the naturally occurring amino acids (Aggeli et al., 2001a). These fibrils are very similar to those formed from HPLC pure $cP_{11}$-4 (Aggeli et al., 2003). The morphological parameters measured from fibrils are shown in Table V.

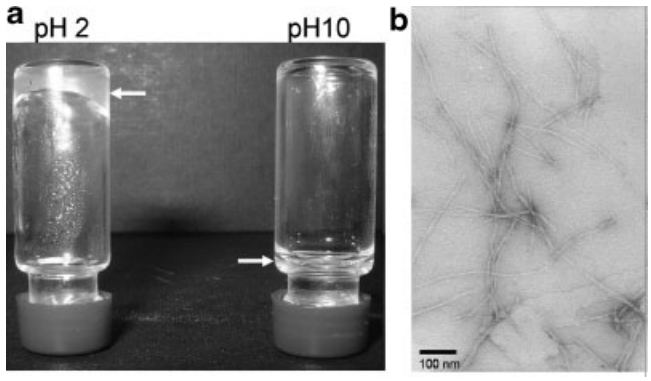


**Figure 4.** Images of $rP_{11}$-4(hsl) hydrogel formation. **a**: A self-supporting hydrogel formed at pH 2 and containing more than 99% water, adhering to the base of an inverted glass vial while at pH 10 the $rP_{11}$-4(hsl) unimer solution sits at the neck of the inverted glass vial. The white arrows indicate the locations of the peptide samples. **b**: TEM analysis of uranyl acetate negative stained samples of rp-HPLC pure $rP_{11}$-4(hsl) reveals self assembly of the peptide into semi-rigid fibrils.

### Characterization of $rP_{11}$-4(hsl) Peptide Secondary Structure

We examined the secondary structure of $rP_{11}$-4(hsl) by CD spectroscopy as a function of peptide concentration in water. Solutions of $rP_{11}$-4(hsl) were prepared at 6.7 mM in water and then induced to form hydrogels at pH 2 by the addition of HCl. Samples for CD analysis were prepared and were quantified by measuring $A_{280}$ after the CD analysis. For reliable analysis of secondary structure, it is necessary to determine the concentration of the solution accurately. Since it is reasonable to assume that the stacking of the peptides by self-assembly into β-sheets would result in the tryptophan not being fully exposed to the solvent, in this study the equation proposed by Pace et al. (1995) for calculating protein concentration was used. For $rP_{11}$-4(hsl) which contains a single tryptophan and no tyrosine or cysteine residues, $\varepsilon$ is 5,500 $M^{-1}\,cm^{-1}$ and the theoretical molecular mass is 1637.7 Da.

Figure 5 shows the molar ellipticity of $rP_{11}$-4(hsl) samples of different concentration at pH 2 in water. For all but the lowest concentration (25 μM) the spectra are consistent with a typical β-sheet secondary structure with minima around 220 nm and maxima at <200 nm. This suggests that the concentration ($c^*$) of the $rP_{11}$-4(hsl) required to form

**Table V.** Comparison of fibril morphologies of purified $rP_{11}$-4(hsl) (this work) and $cP_{11}$-4 (Felton, 2005).

| Sample | $w_w$ (nm) | $w_n$ (nm) | $h$ (nm) | $l$ (μm) | $L$ (μm) |
|---|---|---|---|---|---|
| rP11-4(hsl) | 6–10 | 2–5 | 140 | 0.1–0.3 | 2 |
| $cP_{11}$-4 | 8–14 | 5–8 | 132–360 | 0.7–1.4 | 1.2–7 |

$L$ = longest fibril contour length, $l$ = persistence length, $h$ = full twist pitch, $w_n$ = width of the fibril at its narrowest point, and $w_w$ = width of the fibril at its widest point.

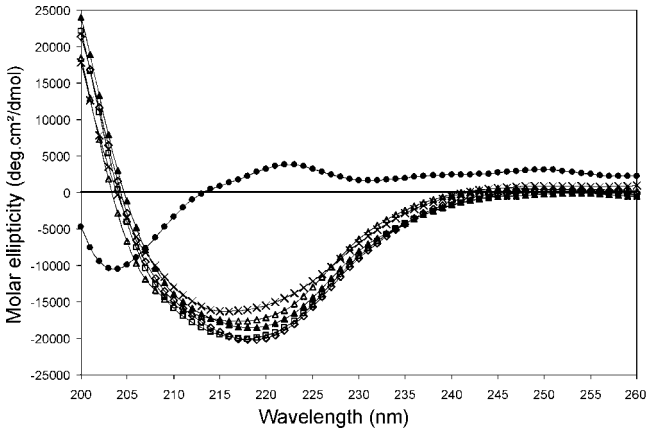


**Figure 5.** Secondary structure analysis of $rP_{11}$-4(hsl). CD spectra displayed as molar ellipticity show the transition to β-sheet in water at pH 2 as a function of peptide concentration; 25 μM (●), 34 μM (▲), 43 μM (×), 50 μM (Δ), 71 μM (◇), and 82 μM (□).

predominantly β-sheet secondary structure is ~30 μM, similar value to that of $cP_{11}$-4 (Aggeli et al., 2003). The gelation behavior, preliminary TEM results and $c^*$ value, showing transition to β-sheet by CD analysis, under the conditions examined indicate that $rP_{11}$-4(hsl) behaves in a similar manner to $cP_{11}$-4.

## Conclusions

We have produced recombinant $rP_{11}$-4(hsl) peptide that self-assembles into self-supporting hydrogels with a fibrillar nanostructure and displays generally similar properties (pH responsive gelation, fibril morphologies, CD profiles) to the chemically synthesized $cP_{11}$-4. High levels of fusion protein were produced following host strain selection and the use of auto-induction; 2.5 g/L of KSI/$rP_{11}$-4(3)/His fusion protein were recovered, both in shake flask and by fermentation, which corresponds to a theoretical yield of 0.53 g $rP_{11}$-4(hsl)/L. The amount of peptide actually purified was 17% of this level indicating this purification step requires further studies to improve recovery. Nonetheless, we purified 90 mg/L of peptide that we believe is the highest level of self-assembling peptide recovery so far reported from a recombinant system. Optimization of various steps including construct redesign, fermentation and peptide purification should further enhance yields of $P_{11}$-4 and be applicable to other self-assembling peptides. Bacterial expression systems continue to offer a valuable route for bioproduction of self-assembling peptides for tissue engineering and other nanoscience applications.

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