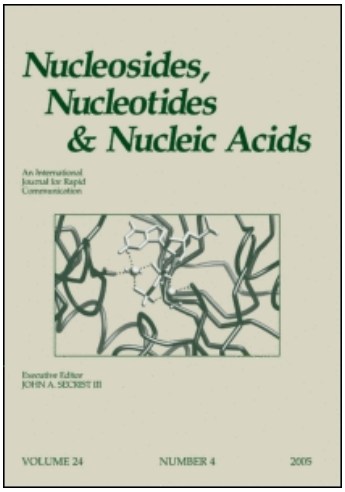

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Solid Phase Synthesis, Cloning and Expression of a Synthetic Gene Encoding a Collagen-Like Peptide

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SOLID PHASE SYNTHESIS, CLONING AND EXPRESSION OF A SYNTHETIC GENE ENCODING A COLLAGEN-LIKE PEPTIDE

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Abstract: A synthetic gene encoding a collagen-like protein (ACMP, artificial cell matrix protein) was designed, constructed and assembled by combined solid phase ligation and PCR-methods. The ACMP-gene (232 bp) was cloned and expressed in an appropiate E. coli expression system. The expressed ACMP (14 kD) was purified by affinity chromatography.

Advances in the preparation, cloning and expression of synthetic genes allow the preparation of materials by means of biotechnology[1]. Genetically programmed protein polymers are produced on the basis of renewable resources, are biocompatible and of controllable biodegradability and can be tailored to an enormous variety of structural arrangements, thus predefining the properties of the materials.

Materials on the basis of cell matrix proteins such as collagen, laminin or fibronectin are of great interest for potential medical applications[2]. They can be used for the construction of three dimensional networks facilitating attachment and growth of specific cells and may play an important role in the wound healing process.

As much as 25% of the protein in mammals is collagen. Various types of collagens are responsible for the functional integrity of tissues, such as bone, cartilage and skin, they also support the structural framework to others such as blood vessels and most organs. The underlying structure that makes them uniquely suited to be the all-purpose construction material of the body is the collagen triple helix. Each of the three α-chains in a collagen consists of repeating tripeptide sequences Gly-X-Y in which the X position is frequently proline and the Y position is frequently hydroxyproline[3].

Our intention is to study the biosynthesis of protein polymers combining structural motifs derived from the triple-helical portion of human collagen type I, with other properties promoting tissue integration and regeneration.

EXPERIMENTAL

Gendesign: The gene designed to code for an artificial cell matrix protein (ACMP) is composed of the following structural domains[4] (FIG.1):

a) Sequences $(GPP)_{10}$, which are known to fold in a triple helical arrangement[5]; alternating with b) a domain defined by the sequence (PVGPRGDS) which is an assembly of a collagenase recognition site and intergrin ligand (RGDS) is expected to allow cell specific adhesion to the polymer[6-8]; and c) a C-terminal $(H)_6$ segment for the purification of the expressed gene product by affinity chromatography[9].

The ACMP gene contains a unique NcoI restriction site at the 5´-end, which provides for the methionine start codon preventing the expression of any additional N-terminal amino acid. The coding domains are separated from each other by a SmaI restriction site, providing for cassette mutagenesis and site directed recombination. The coding sequence is terminated by the presence of a double stop codon, followed by a PstI restriction site.

The $(T)_{12}$ sequence serves as a spacer for the solid phase synthesis. Restriction with NcoI releases the gene from the polymer support.

The sequence (CGT) is coding for arginine and allows the removal of the $(His)_6$ fusion peptides by digestion with carboxypeptidase A.

Synthesis: Oligonucleotides (ACMP 1-ACMP 8, s. FIG.1) were synthesized in a DNA synthesizer (Pharmacia Gene Assembler Plus) according to the phosphoramidite method[10]. The oligonucleotide ACMP 7, which is 5´-biotinylated and ACMP 8, were annealed and immobilized to streptavidin coated magnetic beads (Dynabeads M-280, Merck). The stepwise solid phase gene assembly[11] was performed by successive addition of further annealed fragments (ACMP 1/2, ACMP 3/4, ACMP 5/6, ACMP 9/10) to the starting gene fragment (ACMP 7/8) and ligation by T4-DNA-ligase[12]. We obtained a gene construct consisting of 232 bp (FIG. 2). For cloning experiments the gene product was amplified by PCR (MJ Research Minicycler)

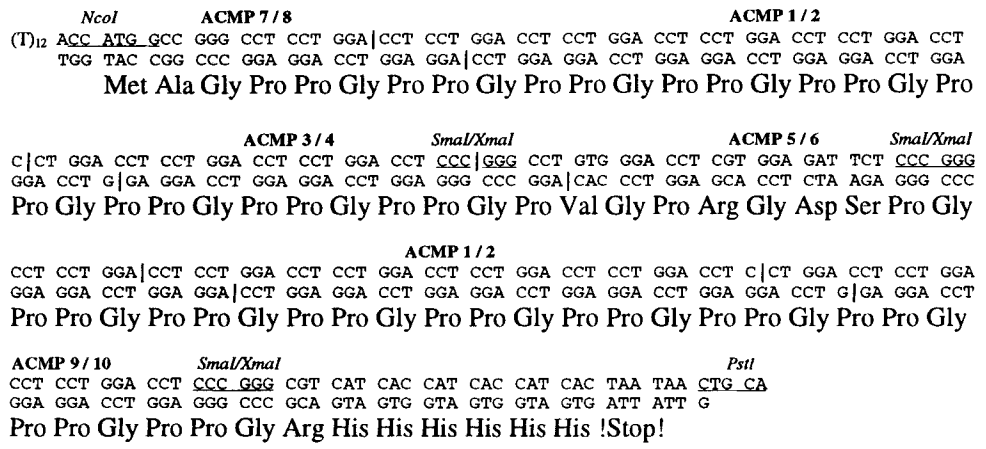

FIG. 1: Sequence of the synthetic ACMP gene and corresponding amino acid sequence. ACMP1-10 indicate the synthetic oligonucleotides used for the construction of the ACMP gene by solid phase gene assembly. The restriction sites for *NcoI*, *PstI*, *SmaI* and *XmaI* are also indicated.

Cloning: The PCR-amplified gene products were ligated with a pGEM-5Zf(+)T-vector (Promega) and transformed into E.coli strain DH5α by electroporation (E.coli Pulser™, Biorad). Sequence analysis was performed with amplification primers using didesoxy-cycle sequencing and dye terminators in an automated sequencer (Perkin-Elmer).

Expression: Recombinant DNA was subcloned in pET-29b(+) (Novagen) for expression in E. coli strain BLR(DE3)pLysS. The pET-29b(+) expression vector contains an S•Tag (recognition site for S-protein alkaline phosphatase) as an N-terminal leader, which allows the detection of the recombinant protein by Western blotting methods.

A single colony was inoculated in 2 ml LB medium containing 30 μg/ml canamycin and incubated overnight at 37°C with shaking. The starter culture was inoculated in 200 ml LB medium containing 30 μg/ml canamycin, 34 μg/ml choramphenicol and 12.5 μg/ml tetracycline and was grown until the density of the cells reaches an OD_{600} of 0.6-0.7 (5-6 hours). Expression of the target protein was induced by addition of 1mM IPTG to the cell culture and incubation for another 2 hours.

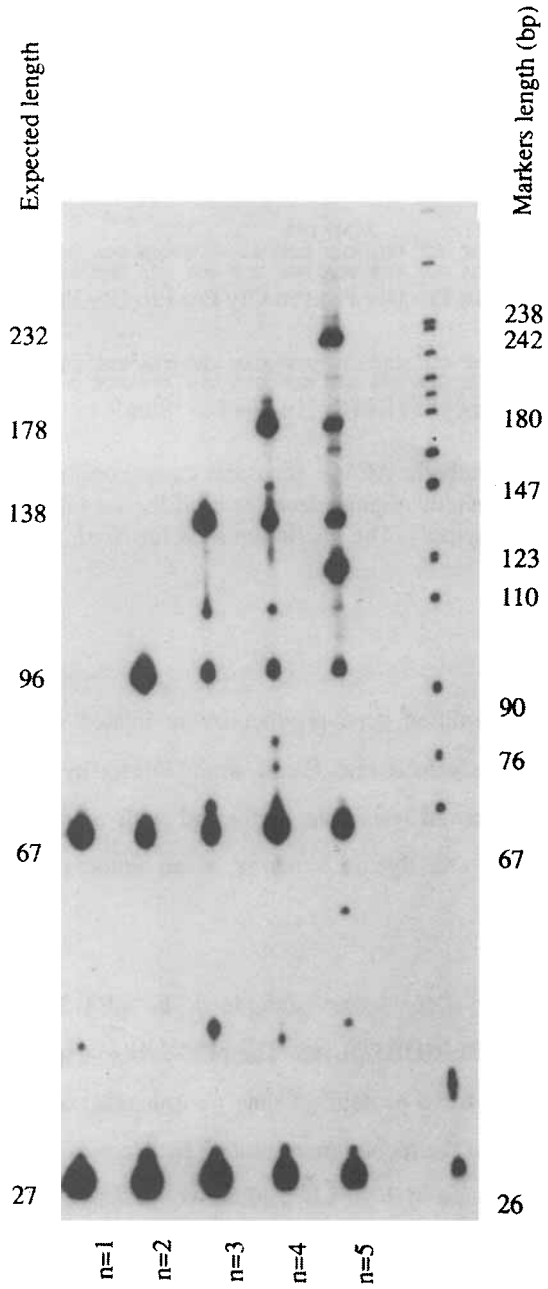

FIG. 2: Synthesis of ACMP gene by stepwise solid phase ligation.
Autoradiograph of a 6% denaturing polyacrylamide gel. (n=1-5: product from ligation step 1-5, M=length marker pBR322/MspI digestion)

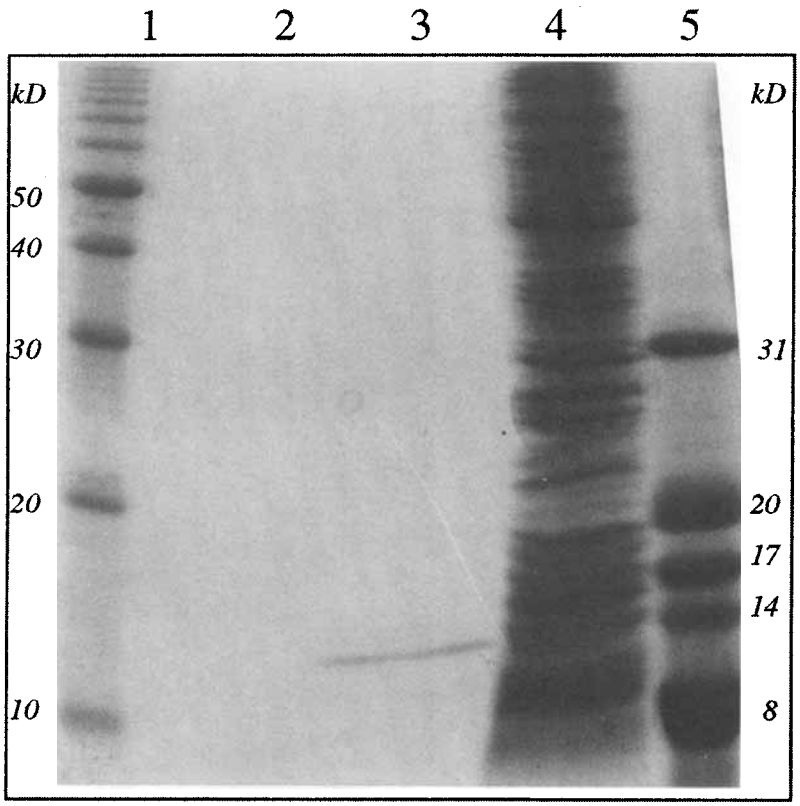

FIG. 3: Analysis of purified ACMP gene product on 16% SDS-PAGE; Coomassie blue stain.
(1) molecular weight protein marker (10 kD ladder); (2) uninduced control, eluate; (3) purified ACMP, eluate; (4) flow through fraction; (5) low range molecular weight protein marker.

Purification: a) Cell lysis: The induced cells were harvested by centrifugation (10 min, 5000 x *g*, 4°C). The cell pellet is resuspended in 1/10 cell culture volume cell-lysis buffer (10 mM Tris HCl, pH 8.0, 1% Triton X-100) and incubated on ice for 30 min. 1 µg RNase and 5 µg DNase were added to the suspension. To reduce the viscosity the cell lysate was passed 3 times through a 0.4mm syringe. The insoluble fraction was removed by centrifugation (20 min, 10 000 x *g*, 4°C).

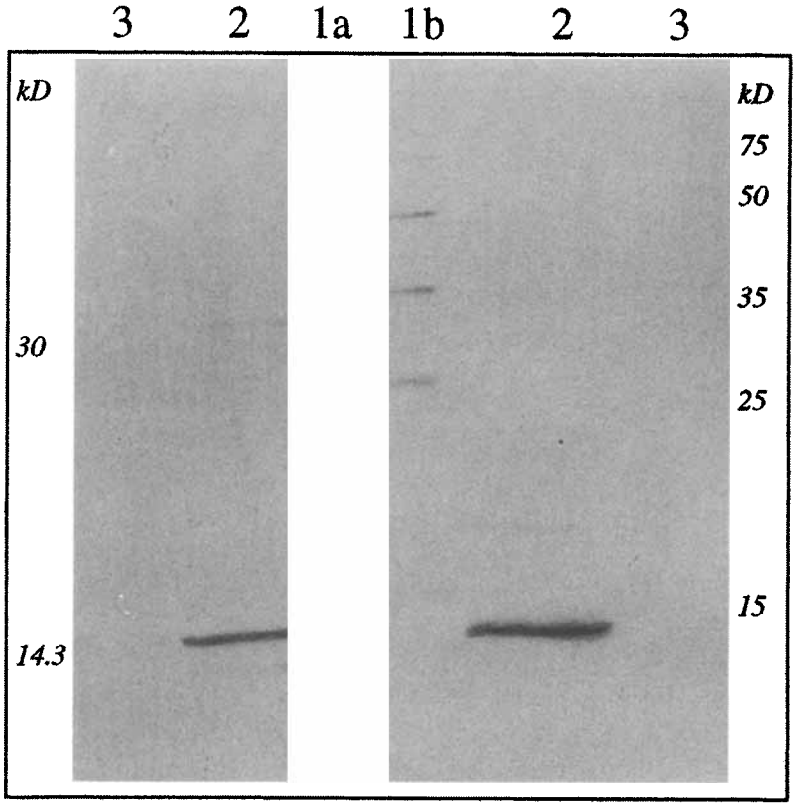

FIG. 4: Western blot of purified ACMP after Ni^{2+} NTA agarose affinity chromatography. (*left*):Detection of the C-terminus via His•Tag. (*right*):Detection of the N-terminus via S•Tag. (1a) molecular weight marker (rainbow marker); (1b) western blot marker; (2) purified ACMP, eluate; (3) uninduced control, eluate.

b) Purification by affinity chromatography

The C-terminal His•Tag sequence of ACMP binds to divalent cations (*e.g.* Ni^{2+}) immobilized on the NTA agarose (Qiagen). After unbound proteins are washed away, the target protein is recovered by elution with imidazole.

350 µl slurry of Ni^{2+}-NTA agarose is added to the soluble fraction of the cell lysate, and the suspension was incubated overnight at room temperature with shaking.

The suspension was transferred to a column, and the resin was washed with 30 ml of 5 mM imidazole in buffer A (10 mM Tris, 300 mM NaCl, 0.25%Triton·X100, pH 8.0), 30 ml of 5mM imidazole in buffer B (10 mM Tris, 300 mM NaCl, 0.25%Triton·X100; pH 6.3) and 60 ml of 50 mM imidazole in buffer B. The purified protein was eluted from the resin with 3 ml of 250 mM imidazole in buffer B.

c) Identification of the expressed protein

The eluted fraction was analyzed by electrophoresis on a 16% SDS-polyacrylamide-gel. A single protein band (FIG. 3) was detected and found to co-migrate with a 14.3 kDa protein standard. For identification the purified expression product was transferred onto nitrocellulose and incubated with the appropriate antibodies (Ni^{2+}NTA conjugate binds to the C-terminal His•Tag and S-protein alkaline phosphatase binds to the C-terminal S•Tag). The target protein was visualized by BCIP and NBT (Western blot, FIG. 4).

RESULTS AND DISCUSSION

Using a solid phase synthesis methods we constructed a gene encoding an artificial cell matrix protein (ACMP). We designed this gene on the structural basis of the triple helical portion of human collagen ($(GPP)_{10}$ sequences) and inserted ligand motives for mammalian cell adhesion and growth (RGDS sequences). A DNA strand of 232 bp was obtained in a completely controlled route of assembly (FIG. 2). Expression and purification of this gene product yields 2-3 μg ACMP protein per ml cell culture. This relatively low yield is probably due to a) the low expression level, which is typical for genes with tandem repeats and b) the formation of insoluble inclusion bodies during expression, which make the extraction and purification of recombinant proteins expressed in E.coli very difficult.

In another approach to synthesize biosynthetic collagens[13] we amplified parts of the gene of human α1-collagen I (768 bp) by PCR and cloned them in expression vectors in an attempt to construct a library of well characterized, expressible collagen-specific DNA fragments.

Both approaches can be linked to yield protein polymers combining the features of human collagen (low immunogenicity and toxicity) with high tensile strength, controlled degradability and properties promoting tissue integration and regeneration.

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