# Chemo-enzymatic synthesis of the human angiogenin gene. Construction of bacterial strains, producers of human angiogenin. Elaboration of a technology for the purification and preparation of angiogenin

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Chemo-enzymatic synthesis of the gene of human angiogenin, a stimulator of blood vessel growth, was performed for the first time. Cloning of the human angiogenin gene in several expressing vector systems was carried out, and several bacterial strains, producers of human angiogenin were constructed. In particular, strains, producers of "angiogenin—β-galactosidase" and "angiogenin—domains of staphylococcal protein A hybrid proteins" were obtained, as well as a bacterial strain efficiently expressing free angiogenin. Large-scale preparation of purified recombinant human angiogenin was accomplished using the *E.coli* JM103 pRITA16 producer strain constructed, and the biological activity of the resulting preparation was studied.

**Key words:** angiogenin, chemo-enzymatic synthesis, cloning, expression, producer strain, hybrid proteins.

## Introduction

Angiogenin is the main protein inducing formation of blood vessels *in vivo*.[1] This protein was found for the first time among the proteins secreted by human carcinoma cells[1] and later it was detected in the liver, in normal human plasma, and in milk.[2—4] Computer analysis of the amino-acid sequence in angiogenin[5] and the corresponding DNA sequence[6] showed that this protein is substantially homologous to mammalian pancreatic ribonucleases. Angiogenin cleaves 18S and 28S RNAs of ribosome subunits into large fragments, but has no effect on some RNAs degraded by pancreatic ribonucleases.[7] Due to the high homology of the amino-acid sequences in angiogenin and in pancreatic ribonuclease and their different substrate specificities, angiogenin is applicable as a model for investigation of the relationship between the structural organization and specificity of enzymes. In addition, some interesting considerations about the possible use of angiogenin in medicine have been reported. First, angiogenin could be useful for accelerating many reparation processes, namely, healing of wounds, burns, and ulcers and, perhaps, also for reparation of postinfarction myocardial disorders.[8] Second, in some cases, inhibition of angiogenin can retard tumor-induced vascular growth and thus deprive the tumor of the necessary blood and nutrition supply.

In the present work, we describe chemo-enzymatic synthesis of the complete human angiogenin gene and expression of the synthetic gene in *E.coli* to give the target product as a component of readily hydrolyzable proteins. Two proteins of this type were obtained: "β-galactosidase—angiogenin" and "IgG-binding domains of staphylococcal protein A—angiogenin". Both proteins were shown to possess angiogenic activity with respect to chorionallantoic membrane of chick embryos. Technology for purification and for large-scale production of recombinant angiogenin is presented. The construction of an *E.coli* strain, a producer of free angiogenin, is described. The results of studies of the biological properties of recombinant angiogenin are presented.

## Chemico-enzymatic synthesis of the human angiogenin gene[9]

The amino-acid sequence of human angiogenin[5] was transformed into a DNA sequence using codons, which are most frequently encountered in intensely expressed genes of *E.coli*.[10] Several codons were replaced by synonymous codons in order to introduce recognition sites for restriction endonucleases into the sequence and to remove the inverted repeats formed with a length of 6 or more bp. Removal of the inverted repeats decreases the probability of formation of pin structures in the mRNA molecule of angiogenin and, therefore, the probability that steric restrictions for the protein-synthesizing apparatus will arise on mRNA during expression of the angiogenin gene in *E.coli*.

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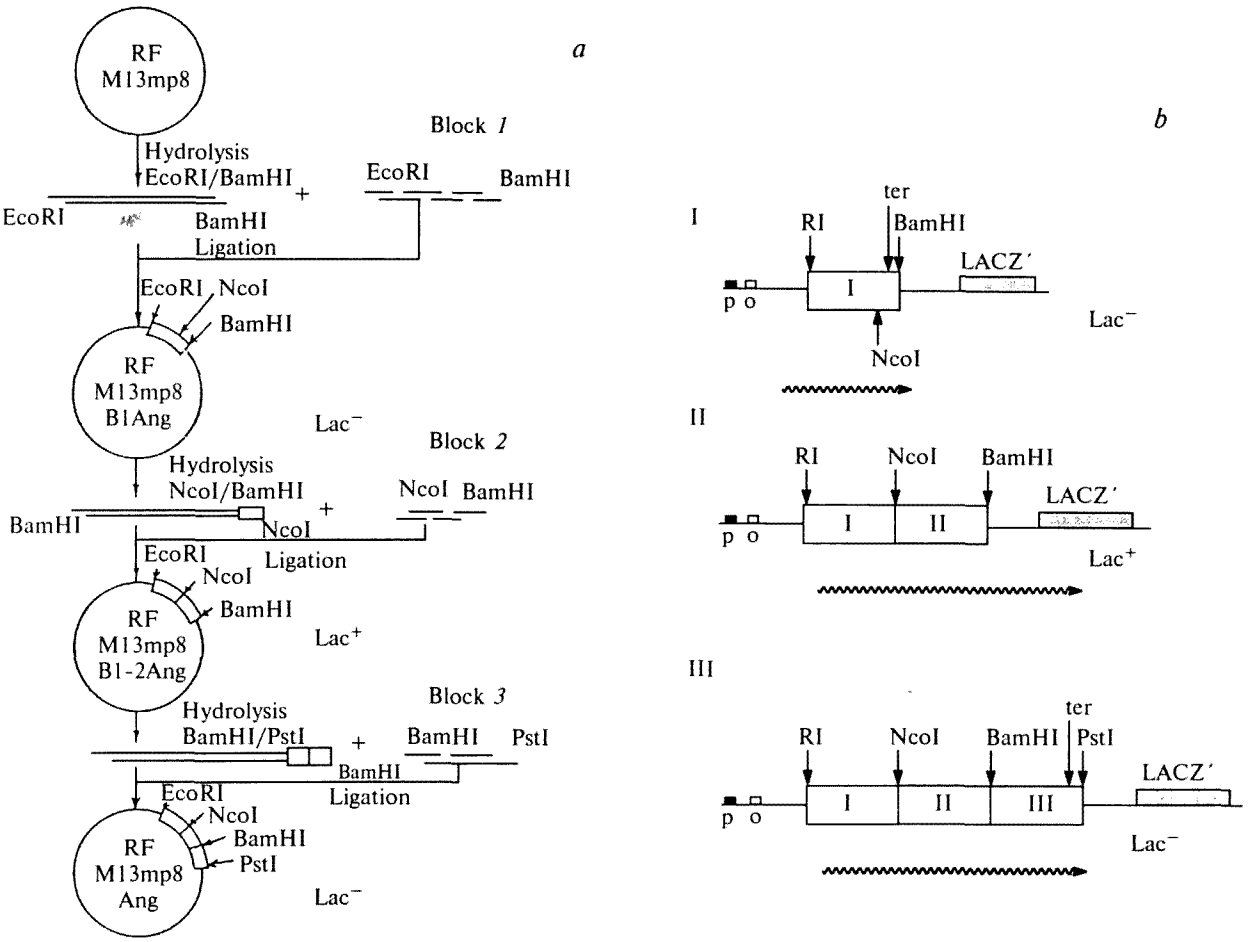


**Fig. 1.** *a*. General scheme for cloning the angiogenin gene and its blocks. *b*. Phenotypic changes associated with assembly of the angiogenin gene in the M13mp8 phage. Translation frames are shown by arrows, sites for cleavage by restriction endonucleases are shown above the scheme. RI is EcoRI; ter is the terminator of translation; p is the lac-promoter; o is the lac-operator. I—III are three successive clonings.[9]

To ensure correct initiation of translation, an ATG codon was introduced ahead of the first codon of angiogenin, and TAA and TGA codons terminating translation were introduced after the C-terminal CCG triplet. Since we planned to clone the angiogenin gene in the polylinker of the M13mp8 phage in parts, the calculated DNA sequence was divided into three blocks by introducing recognition sites for NcoI and BamHI restrictases into the encoding part of the gene. In addition, an XhoI site was introduced at the beginning of the encoding part of the gene, an SalGI site was introduced at its end, and the whole sequence was flanked with EcoRI and PstI sites.

The general scheme for cloning the angiogenin gene is shown in Fig. 1, *a*. According to the scheme accepted for the division of the polynucleotide chain into blocks, cloning of the first block in the polylinker of the M13mp8 phage should lead to termination of synthesis of the β-galactosidase α-peptide of M13 phage, cloning of the second block should restore the translation frame, and cloning of the third block should again terminate translation before synthesis of the α-peptide begins (Fig. 1, *b*). This scheme made it possible to select recombinant phages during cloning of each subsequent block according to the variation of the phenotypes of phage colonies:

$$Lac^+ \rightarrow Lac^- \rightarrow Lac^+ \rightarrow Lac^-.$$

The division of each block into oligonucleotides was carried out using a special computer program, which ensured minimization of the energy of formation of unplanned duplexes, and, hence, the minimum number of by-products during ligase cross-linking of oligonucleotides (the program was kindly provided by A. E. Nikulin, the State Scientific Center of Virology and Biotechnology "Vektor"; all calculations were carried out using a computer at the State Computer Center of the Siberian Branch of the RAS).

The full structure of the synthetic angiogenin gene with the division into blocks for cloning, the fragments for ligase crosslinking, and the initial synthetic oligonucleotides are presented in Fig. 2.


**Fig. 2.** Nucleotide sequence in the three blocks used for successive cloning. Oligonucleotides are denoted by arrows, the DNA fragments after ligation of oligonucleotides are marked by brackets. Amino-acid sequences are shown above the nucleotide sequences, and the sites for restriction endonucleases are shown below the sequence.[9]

Forty-three oligonucleotides with lengths of 15 to 22 bp were synthesized on a "Viktoriya-4M" serial automatic synthesizer (produced by the Design Bureau for Special Electronics and Automatic Instrumentation of the Siberian Branch of the RAS) using the phosphitamide method.[11] When synthesis was completed and all of the protective groups, except for the 5′-end dimethoxytrityl group, had been removed, the product was isolated by reversed-phase HPLC, the dimethoxytrityl group was removed, and the compound was rechromatographed on a column with a reversed-phase support.[12]

The oligonucleotides synthesized were phosphorylated at the 5′-end using T4-polynucleotide kinase, mixed in equimolar amounts, and treated with DNA-ligase of the T4 phage. Each chain of a block fragment was cross-linked separately,[13] and the reaction products were separated in polyacrylamide gel and isolated by electroelution.

The DNA fragments thus formed were mixed in equimolar amounts and ligated using a vector prepared beforehand (see Fig. 1, *a*). In the cloning of block *1*, phages giving white plaques on the JM103 indicator lawn were selected, in the cloning of block *2*, phages giving blue plaques were selected, and in the cloning of block *3*, phages giving white plaques were selected again. The presence of the insert in the phage at each stage was confirmed by fusion with the corresponding initial nucleotides. The primary DNA structures of fused phages were analyzed[14] after assembly of the complete gene. Along with the versatile 17-membered oligonucleotide,[14] oligonucleotides synthesized for assembly of the structural part of the angiogenin gene were used as primers (see Fig. 2, oligonucleotides NN 38, 30, 22, and 14). This permitted the structures of the fragments under analysis to be established more reliably.

During sequencing of DNA, it was found that the fused phage contains the synthesized human angiogenin gene, whose structure corresponds completely to the planned structure.

Thus, a DNA fragment containing 389 nucleotide pairs and encoding the complete amino-acid sequence of human angiogenin was synthesized using chemical and enzymatic techniques. The presence of the XhoI site at the beginning and the SalGI site at the end of the coding part of the fragment allows its use in various vector systems. The fragment was initially cloned in the

M13mp8 phage, which ensured the possibility of investigating the structural and functional organization of angiogenin by the method of oligonucleotide-directed mutagenesis.[15]

Recently,[16] chemo-enzymatic synthesis of the human angiogenin gene has been carried out according to a different scheme; synthesis and cloning of the complementary DNA (cDNA) of human angiogenin were also reported.[6,17,18]

## Expression of the synthetic human angiogenin gene within "β-galactosidase—angiogenin"[19] and "angiogenin—staphylococcal protein A domains"[20] chimeric proteins

In order to obtain angiogenin in amounts sufficient for medical and biological assays, we studied genetic engineering producers of human angiogenin. The first stage of these studies dealt with expression of the synthetic angiogenin gene in *E.coli* to give a chimeric protein "β-galactosidase—angiogenin" and subsequent chemical abstraction of angiogenin.

Despite the fact that a large number of studies devoted to expression of synthetic and natural genes in *E.coli* have been published,[21] no absolutely universal expression systems have yet been developed. For expression of each particular gene, a special optimal combination of regulatory elements is normally chosen. At present, systems ensuring the production of chimeric proteins can be considered to be rather versatile. If we take into account the accessibility of expressing systems and the possibility of abstracting the target product from the chimeric protein synthesized, the widely used pUR290 plasmid seems quite attractive for this purpose.[22] We used this plasmid to obtain the "β-galactosidase—angiogenin" chimeric protein.[19,20] The DNA of the pUR290 plasmid was hydrolyzed with SalGI/Hind III restrictases, and the products were mixed in a ratio of 1 : 3 with the restriction fragments of the DNA of M13-Ang phage,[9] which were first hydrolyzed with XhoI/Hind III restrictases. The resulting mixture was ligated, and *E.coli* JM 103 cells were transformed by the ligation products. The transforms resistant to ampicillin were analyzed by fusion and by restriction analysis for the presence of the insert, corresponding in its molecular weight to the fragment that encodes angiogenin. Subsequently, one of the selected clones, which was called K8 (pUR290-Ang), was used (Fig. 3). Analysis by gel electrophoresis of the total cell proteins of the K8 clone with induction of the lac-promoter of the pUR290-Ang plasmid by isopropyl thiogalactoside (IPTG) showed that the molecular weight of the protein being induced is higher than that of β-galactosidase (Fig. 4) and, in all probability, this protein is the "β-galactosidase—angiogenin" chimeric protein.

The polylinker section of the pUR290 plasmid and the corresponding section of the pUR290-Ang recombinant plasmid contain the ...GATCCG... nucleotide se-

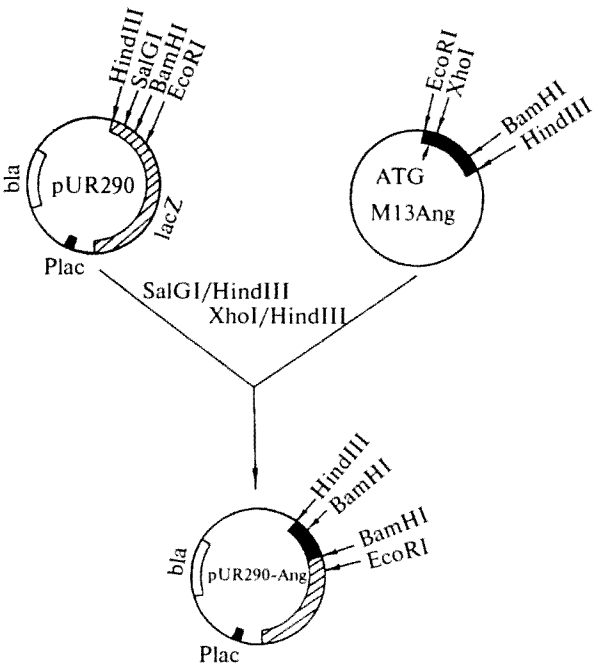


**Fig. 3.** Scheme for the construction of the pUR290-Ang plasmid. Sections of the structural genes of angiogenin are in black, those of β-galactosidase are hatched.[19]

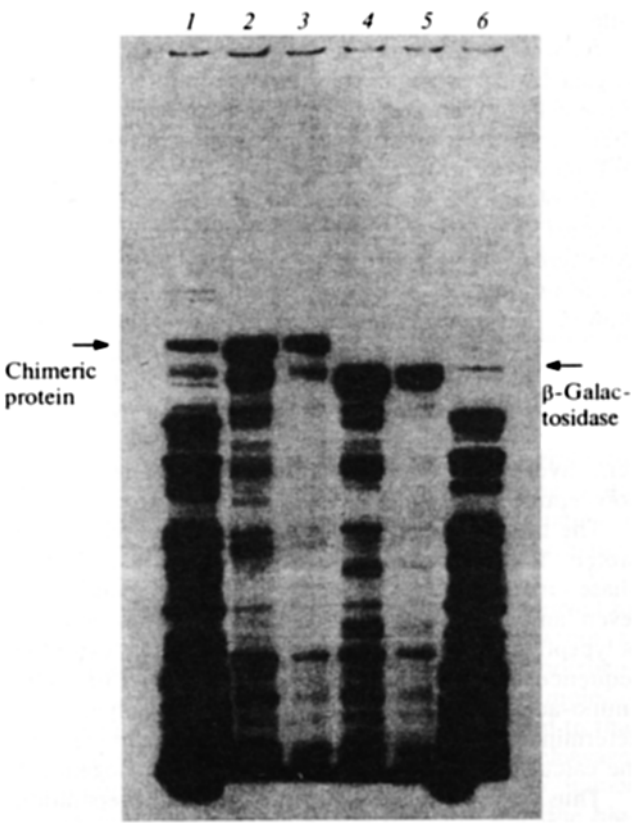


**Fig. 4.** Electrophoresis in 7% PAAG according to Laemmly of total cell proteins from *E.coli* strains carrying pUR290-Ang (*1—3*) or pUR290 (*4—6*) plasmids. Growth without induction by IPTG (*1*, *6*); growth for 5 h after induction by IPTG (*2—5*).[19]

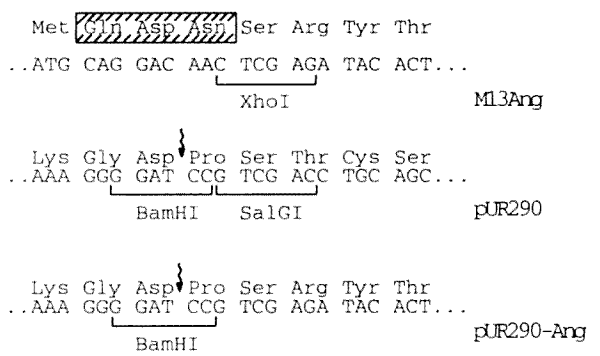


**Fig. 5.** Fragments of amino-acid sequences and the corresponding nucleotide sequences of plasmids used in this work. The amino acids at the N-end of the angiogenin chain, deleted in the chimeric protein, are hatched; the site of the acid-labile peptide bond is marked by an arrow.[19]

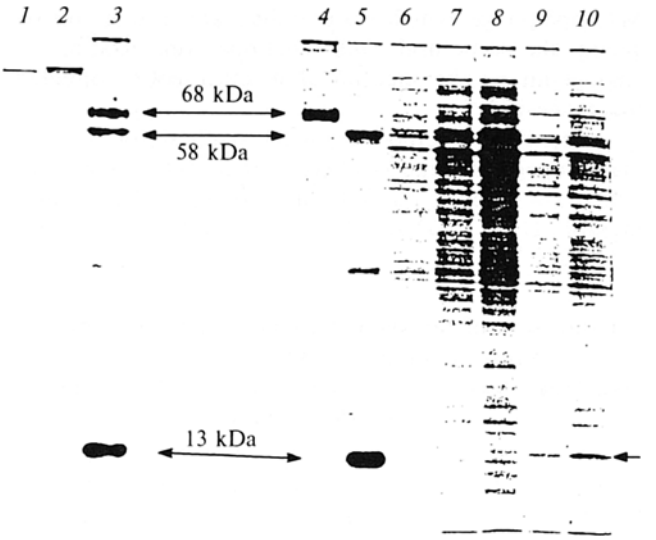


**Fig. 6.** Electrophoresis in 15% PAAG of the polypeptides formed upon hydrolysis of "insertion bodies" isolated from the *E.coli* strains containing pUR290 and pUR290-Ang plasmids. "Insertion bodies" of *E.coli* with pUR290 plasmid (β-galactosidase) prior to hydrolysis (*1*); the same after hydrolysis with 10% acetic acid (*6—8*); insertion bodies of *E.coli* with the pUR290-Ang plasmid prior to hydrolysis (*2*); the same after hydrolysis with 10% acetic acid (*9*, *10*); protein molecular weight markers (*3—5*).[19]

quence, encoding the Asp—Pro amino-acid sequence (Fig. 5), which can be cleaved by mild acid hydrolysis.[23] In the chimeric protein obtained, the Asp—Pro sequence should be located at the boundary between the polypeptides corresponding to β-galactosidase and angiogenin; therefore, one may expect that the polypeptide corresponding to angiogenin could be abstracted by mild acid hydrolysis.[23]

As was also reported in a number of studies,[21] the "β-galactosidase—angiogenin" chimeric protein exists in *E.coli* cells as inclusion bodies, whose isolation results in effective purification of the chimeric protein (up to 70—80% purity) (Fig. 6).

To obtain the polypeptide which corresponds in its structure to angiogenin, the Asp—Pro peptide bond was hydrolyzed with 70% formic acid. Electrophoretic analysis of the products of acid hydrolysis of bacterial proteins showed that hydrolysis did occur, but it yielded no polypeptide with the molecular weight of 13 kDa calculated for angiogenin. Therefore, the initial material was cleaved in 6 *M* guanidine hydrochloride in a pyridinium acetate buffer (pH 2.5). Under these conditions, limited acid hydrolysis was accomplished and the desired polypeptide was obtained.

The target product was isolated from the mixture of protein fragments by chromatography on a reversed-phase sorbent, Polisil ODS-300. The N-end sequence of seven amino acids incorporated in the homogeneous polypeptide thus obtained corresponded to the expected sequence: Pro—Ser—Arg—Tyr—Trp—His—Phe. The amino-acid composition of the isolated polypeptide, determined after hydrolysis, was in good agreement with the calculated amino-acid composition of angiogenin.[24]

Thus, the system used allows rather easy preparation of the "β-galactosidase—angiogenin" chimeric protein. The arrangement of nucleotides which encode the Asp—Pro amino-acid sequence in the polylinker section of the pUR290 plasmid makes it possible to abstract the target polypeptide for almost any cloning scheme in pUR290. Apparently, the conditions described for the cleavage of the Asp—Pro bond can also be used for other proteins fused with β-galactosidase: according to our data, the Asp—Pro sequence encoded by the polylinker is the most susceptible to acid hydrolysis of the chimeric protein. The Asp—Pro bond in other polypeptides is also cleaved fairly selectively under similar conditions;[23] however, this bond is quite uncommon in natural peptides.

As noted above, French researchers described[16] expression of the synthetic angiogenin gene in *E.coli*. Angiogenin was prepared using a specially constructed vector, pXL 694. In that study, the recombinant protein accounted for 2—5% of the total cell protein, whereas in our case, the hybrid protein accounted for 15—20% of the total cell protein (or 1.5—3%, if calculated for angiogenin). Thus, the amounts of angiogenin synthesized in *E.coli* using the two methods under consideration are comparable.

It should be noted that the amino-acid sequence in the polypeptide resulting from acid cleavage of the chimeric protein differs from that in natural angiogenin in the N-end sequence of amino acids and contains a proline residue instead of Gln-Asp-Asn tripeptide (see Fig. 5). However, this modification seems insignificant, at least as regards the ribonuclease activity of the protein. In fact, the structure of the N-end fragment varies in the series of ribonucleases homologous to angiogenin, but this barely affects their functional characteristics.[25]

In bioassays carried out using the chorionallantoic membrane of chick embryos, we demonstrated that the "β-galactosidase—angiogenin" hybrid protein possesses clear-cut angiogenic activity, although the minimum quantity of the protein inducing angiogenesis is much larger than that in the case of natural angiogenin.

In our attempt to improve and simplify the procedure of purification of the hybrid protein containing the amino-acid sequence of angiogenin, we used the "affinity handle" approach, which was employed successfully for purification of various hybrid proteins both in the laboratory and on the industrial scale.[26—28]

The strain-producer of "IgG-binding domains of staphylococcal protein A—angiogenin" chimeric protein was obtained by introducing pRITA16 and pRITC$_1$857 plasmids into the *E.coli* K12JM103 strain.[20] The DNA sequence that encodes angiogenin was modified by insertion of an oligonucleotide duplex into the EcoRI/XhoI sites in such a way that two codons (GATCCG) corresponding to Asp—Pro were formed in the frame just before the sequence encoding angiogenin. This modified DNA was ligated into the EcoRI/PstI sites of the pRIT33 plasmid, thus forming an open read-out frame, "IgG-binding domains of protein A—Asp—Pro—angiogenin". The recombinant plasmid was denoted pRITA16. The physical map of this plasmid is presented in Fig. 7. The pRITA16 plasmid directs the synthesis of the domains of protein A—angiogenin chimeric protein in the cell; intense transcription is ensured by the Pr promoter of the λ phage, while intense translation is provided by the SD-sequence of the CRO gene of the λ phage.

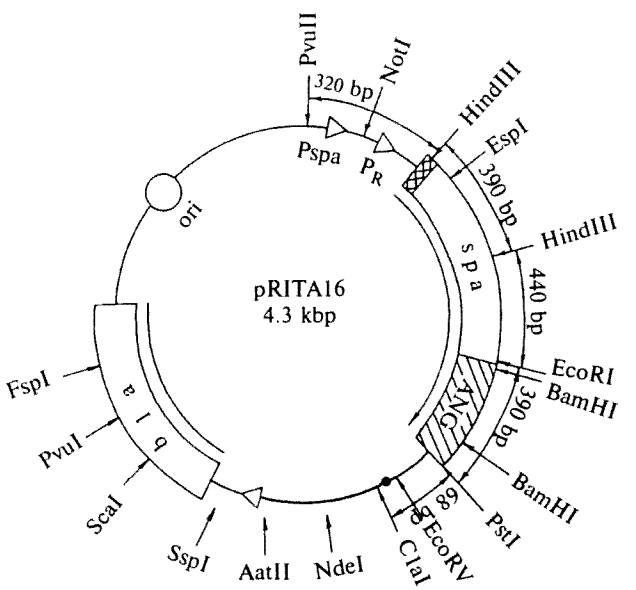


**Fig. 7.** The pRITA16 plasmid directing synthesis of the "IgG-binding domains of protein A—angiogenin" hybrid protein.

▨ is a fragment of $\lambda_{cro}$ gene, ▨ is the sequence encoding angiogenin, ▭ is a fragment of the gene encoding protein A, and ◯ is the terminator of transcription.

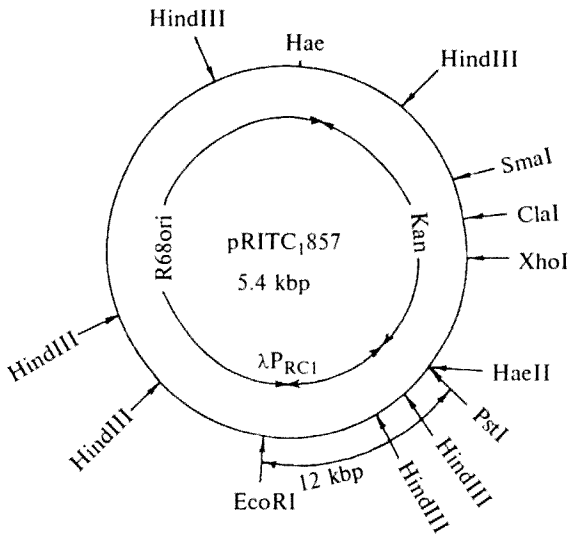


**Fig. 8.** The pRITC$_1$857 plasmid directing synthesis of C$_1$857 protein, a repressor of $\lambda P_R$ promoter.

The plasmid thus obtained was introduced into *E.coli* JM103 cells containing the pRITC$_1$857 plasmid (its physical map is shown in Fig. 8), ensuring synthesis of thermally labile C$_1$857 repressor, which suppresses transcription from the Pr promotor. As a result, synthesis of this repressor occurs in the cells of the *E.coli* pRITA16 strain at 32 °C, whereas an increase in the temperature to 42 °C leads to depression of the Pr promoter and, consequently, to intense transcription and translation, and to the formation of "IgG-binding domains of protein A—angiogenin" hybrid protein. The pRITA16 plasmid forms ~300 copies per cell. The IgG-binding fraction of the soluble extract of *E.coli* contained a protein with the expected molecular weight (~43 kDa), together with proteins that had molecular weights of 30—40 kDa and probably resulted from proteolytic degradation of the hybrid protein.

It was found that the IgG-binding fraction from *E.coli* RITA16 strain exhibits angiogenic activity with respect to chorionallantoic membrane of chick embryos, even when the quantity of protein applied on the membrane is only 20 μg. Neither IgG-binding domains of protein A nor the protein A itself possessed such high activity.

The hybrid protein that we obtained was cleaved by acid hydrolysis with 70% formic acid carried out at 42 °C for 16—48 h. Hydrolysis yielded several polypeptides with the expected molecular weights. The molecular weight of one of these products corresponded to that of angiogenin. It is noteworthy that after cleavage, the minimum quantity of protein needed to induce angiogenesis was 200 μg, which indicates that the biological activity had substantially decreased.

The technological scheme for production of recombinant angiogenin based on the *E.coli* pRITA16 strain is presented below.

```
                EcoRI                    XhoI
5'-CTGCCAGAGGGAGAATTCG ATG CAG GAC AAC TCG AGA TAC ACT CAC TTT CTG ACT CAG 39
                       Met Gln Asp Asn Ser Arg Tyr Thr His Phe Leu Thr Gln

   CAT TAC GAT GCT AAA CCA CAG GGC CGC GAC GAC CGT TAT TGC GAA TCT ATT ATG 93
   His Tyr Asp Ala Lys Pro Gln Gly Arg Asp Asp Arg Tyr Cys Glu Ser Ile Met

   CGC CGC CGC GGT CTG ACC TCT CCA TGC AAA GAC ATC AAC ACT TTC ATC CAT GGT 147
   Arg Arg Arg Gly Leu Thr Ser Pro Cys Lys Asp Ile Asn Thr Phe Ile His Gly

   AAC AAA CGT TCT ATC AAA GCT ATC TGC GAA AAC AAA AAC GGC AAC CCG CAC CGC 201
   Asn Lys Arg Ser Ile Lys Ala Ile Cys Glu Asn Lys Asn Gly Asn Pro His Arg

   GAA AAT CTG CGT ATC TCT AAA TCT TCT TTC CAG GTC ACT ACT TGC AAA CTG CAC 255
   Glu Asn Leu Arg Ile Ser Lys Ser Ser Phe Gln Val Thr Thr Cys Lys Leu His

        BamHI
   GGT GGA TCC CCG TGG CCG CCA TGT CAG TAC CGT GCT ACT GCT GGC TTC CGT AAC 309
   Gly Gly Ser Pro Trp Pro Pro Cys Gln Tyr Arg Ala Thr Ala Gly Phe Arg Asn

   GTT GTT GTT GCA TGC GAA AAC GGC CTG CCG GTT CAC TTG GAC CAG TCT ATC TTC 363
   Val Val Val Ala Cys Glu Asn Gly Leu Pro Val His Leu Asp Gln Ser Ile Phe

      SalI                PstI        HindIII
   CGT CGA CCA TAA TGA CTGCAGGCATGCAAGCTT-3'                              397
   Arg Arg Pro *** ***
```

**Fig. 9.** Nucleotide sequence of the angiogenin gene within pRAngT plasmid.

### Construction of the producer strain efficiently expressing free human angiogenin[29]

Systems for the expression of a target product as a component of hybrid proteins have some advantages but also serious drawbacks. The latter are mostly associated with the fact that chimeric protein needs to be chemically treated in order to abstract the desired polypeptide. We attempted to accomplish direct expression of the gene under consideration in *E.coli* cells within a plasmid vector. The fact that genes of human interleukin-2 (IL-2) and its mutant analogs, as well as some other genes, controlled by the *Proteus mirabilis* recA promoter, have been expressed successfully[30] made it possible to expect that this problem would be solved by using the expression system under consideration.

We used the pRTU1 plasmid, obtained earlier from the pRIL3 plasmid,[30] as a vector making it possible to synthesize human angiogenin as an individual protein in *E.coli* cells. This plasmid contains the *Proteus mirabilis* recA promoter, the trp-A terminator of *E.coli* transcription with the preceding polylinker section, meant for gene cloning, and the ampicillin-resistance gene. The synthetic gene of angiogenin[9] was incorporated into the polylinker section of the pRU1 plasmid controlled by the recA promoter. The nucleotide sequences of the incorporated gene and adjacent sections of the vector in the resulting pRAngT plasmid were determined using the Maxam—Gilbert method (Fig. 9). The structure of this plasmid (Fig. 10) is such that under the control of the *Proteus mirabilis* recA promoter, an individual protein encoded by the synthetic angiogenin gene is synthesized. Effective termination of transcription is accomplished at the ρ-independent terminator of the *E.coli* tryptophan operon located in the 3′-flanking region of the angiogenin gene within the expression plasmid that was obtained. In the cells of the *E.coli* VL1222 strain possessing reduced ability for intracellular proteolysis, after transformation with the pRAngT plasmid, angiogenin is expressed following induction by nalidixic acid or mitomycin C (Fig. 11). The recombinant protein is accumulated within non-soluble inclusion bodies. The immunospecificity of the product of expression was proved by the immunoblot method using monoclonal antibodies to human angiogenin.

The recombinant *E.coli* JM103 pRITA16 and *E.coli* VL1222 pRAngT strains were certified and deposited in the All-Russian collection of industrial microorganisms.

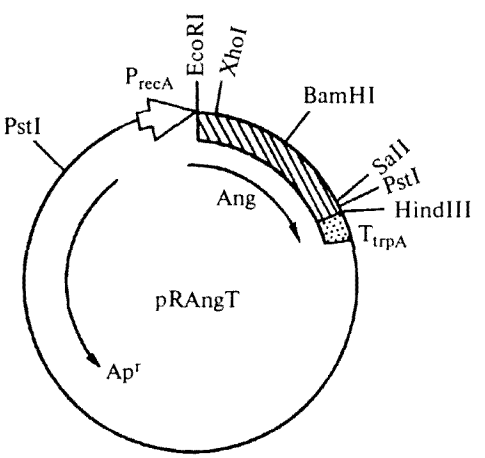


**Fig. 10.** Structure of the pRAngT plasmid. The large arrow marks the promoter of the *Proteus mirabilis* recA-protein, the sloping hatching denotes the angiogenin gene, and the dots denote trpA, the terminator of transcription.

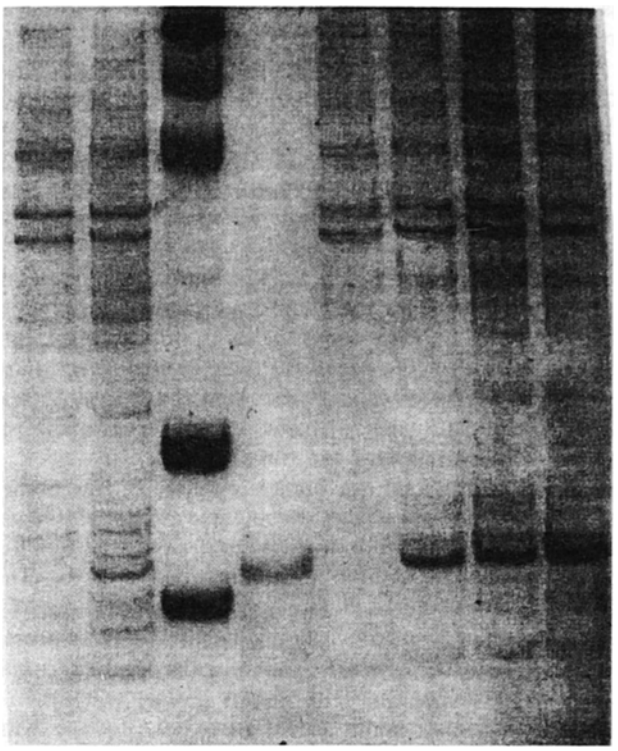


**Fig. 11.** Expression of the human angiogenin gene in the *E.coli* VL1222 cells transformed by pRAngT plasmid. Electrophoretic pattern, 15% SDS-PAAG. Cell lysate without induction of recA promoter (*1*); cell lysate after 6 h of induction by mitomycin C (*2*); set of protein molecular weight markers (from the top down: 132; 94; 66; 20.1; and 13.7 kDa) (*3*); angiogenin (produced at the Institute of Biotechnology "Fermentas", Vilnyus, Lithuania) (*4*); precipitate resulting from destruction of cells and centrifugation (without induction) (*5*); the same, after 2, 6, and 12 h of induction, respectively (*6*, *7*, *8*).

## Development of technology for isolation and purification of the "β-galactosidase—angiogenin" and "protein A domains—angiogenin" recombinant proteins from microbial biomass

Technology for the large-scale production and purification of recombinant human angiogenin was developed at the Institute of Biotechnology "Fermentas" (Lithuania).

The technological scheme for preparation of the biomass of the producer of "β-galactosidase—angiogenin" (pUR290-Ang plasmid) includes the following stages: (1) cultivation of the strain in a medium consisting of the following components (manufactured in Russia): aminopeptide (22 mg $mL^{-1}$), bakers yeast hydrolyzate (5 g $L^{-1}$), NaCl (10 g $L^{-1}$), and ampicillin (50 mg $L^{-1}$); (2) inoculation of a laboratory fermenter containing 10 L of said medium with 10% of the inoculate and cultivation in the fermenter up to an optical density $D_{280}$ of 3.0±0.3; (3) induction of synthesis of the hybrid protein by IPTG, manufactured in Russia, (200 mg $L^{-1}$) over a period of 3.5±0.5 h.

Purification of the "β-galactosidase—angiogenin" hybrid protein was carried out from "inclusion bodies". The "inclusion bodies" were isolated as described previously,[21] the protein was extracted by guanidinium chloride, which was then removed by dialysis. The chimeric protein thus obtained was converted into water-soluble form by dissolution in 6 *M* urea, adsorption on DEAE-cellulose, and elution with a phosphate buffer solution containing 1 *M* NaCl (without urea). The resulting protein samples with a concentration of 2 mg $mL^{-1}$ were used in the subsequent work.

The "β-galactosidase—angiogenin" hybrid protein induced blood vessel growth in experiments with chorionallantoic membrane of chick embryos.

As noted above, the "β-galactosidase—angiogenin" hybrid protein was cleaved at the acid-labile Asp—Pro bond. Optimal cleavage was carried out in a 6 *M* solution of guanidine hydrochloride using 10% acetic acid with pyridine added to pH 2.5, or in a 6 *M* solution of guanidine hydrochloride without addition of pyridine.

Cleavage was carried out immediately after extraction of the protein from the "inclusion bodies" with guanidine hydrochloride. The mixture of proteins containing angiogenin was transferred from the solution in guanidine hydrochloride into a 6 *M* solution of urea by ultrafiltration, and angiogenin was then purified on DEAE-cellulose in a 0.02 *M* phosphate buffer with 6 *M* urea (pH 7.5); the ballast proteins were retained on the column, while angiogenin was not sorbed.

The biochemical activity of the "β-galactosidase—angiogenin" hybrid protein in the form of "inclusion bodies" insoluble in water and also in solution was assayed using chorionallantoic membranes of chick embryos. The biological activity, specifically, the ability to induce neovascularization, was also exhibited by the polypeptide, obtained by excision from the hybrid protein, which consisted of the angiogenin amino-acid sequence containing a proline residue instead of the three N-end amino acids. Thus, the genetic engineering strategy described in this paper is quite suitable for the production of a specimen inducing angiogenesis. Substantial losses accompanying purification of angiogenin obtained by cleavage of the hybrid protein, and the tendency of the hybrid protein converted into a water-soluble form for aggregation should be regarded as drawbacks of this approach. The simplicity and substantial versatility of this scheme is among its obvious advantages. The fact that the protein isolated from the "inclusion bodies" in water-soluble form exhibits angiogenic activity is also an advantage of the method. Probably, proteins of this type can serve as the basis for development of a stable product, which would exhibite prolonged action without any need to immobilize the protein.

A schematic diagram of preparation of recombinant angiogenin using the *E.coli* pRITA16 strain is shown in Fig. 12. The domains of "protein A—angiogenin" hybrid

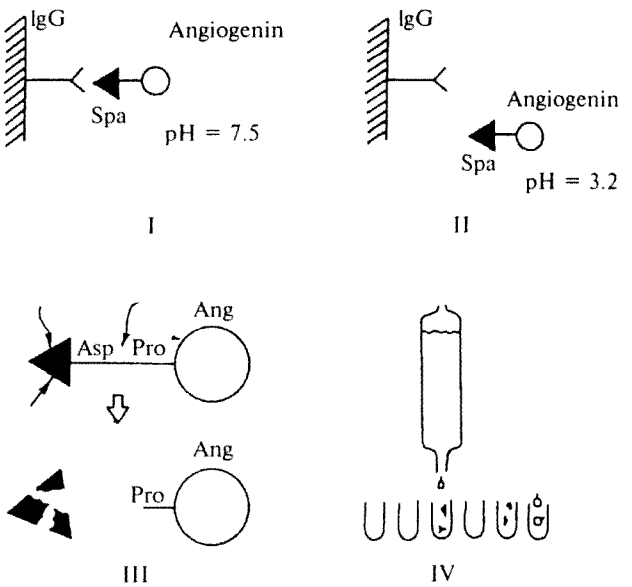


**Fig. 12.** General scheme for production of Pro-angiogenin from cell proteins containing "domains of protein A—angiogenin" hybrid protein. I, binding; II, elution; III, cleavage; IV, chromatography.

protein is sorbed on a column with IgG-sepharose and can be eluted with 0.3 *M* acetic acid. This protein is cleaved during mild hydrolysis under conditions where Asp—Pro bonds are cleaved preferentially and Pro-angiogenin is thus released (75% $CH_3COOH$, 37 °C, 48 h).

Subsequently, the cleaved protein is purified by chromatography on SH-Sepharose. The proteins are applied as solutions in 0.01 *M* phosphate buffer (pH 6.6) containing 0.25 *M* NaCl. The target protein is eluted with a 0.1 *M* glycine buffer solution (pH 10.6) containing 0.2 *M* NaCl. The electrophoretic purity of the sample of angiogenin thus obtained is 85—90%.

### Study of the biological properties of the recombinant purified proteins, "β-galactosidase—angiogenin" and "protein A domains—angiogenin"

We analyzed the immunomodulating activity of the recombinant angiogenin obtained from *E.coli* pRITA16 strain and tested the Pro-angiogenin preparation for its acute and chronic toxicity together with the Institute of Physiology of the Siberian Branch of the Russian Academy of Medical Sciences.[31]

These experiments demonstrated for the first time the immunomodulating function of angiogenin, which is manifested in its dose-dependent opposite effect on the antigen-induced activation of cells of the immune system. The results obtained suggest that in the case of angiogenin-dependent tumors, the factor under investigation not only favors vascularization of neoplasms, but is also used by the latter as a means for immune protection.

In cooperation with the Institute of Therapeutics, we carried out a series of studies of the effects of angiogenin on an experimental model of acute sores. The purpose of these experiments was to study the characteristic features of the effect of angiogenin on reparative and regenerative processes in rat skin after experimental scalding.

It was found that administration of recombinant angiogenin does not accelerate sore healing in white rats. In addition, the healing of sores and the formation of cicatrices after the administration of angiogenin have a specific character. This is manifested as physiological skin regeneration, accompanied by almost complete recovery of skin structure and function.

These results confirm the known data on the ability of angiogenin to induce complete vascularization in tissues,[32,33] which leads to better physiological healing of damaged skin without formation of a cicatrix. The results that we obtained indicate that elaboration of a medical preparation based on angiogenin would be promising for treating extensive skin damage and for more complete skin healing. The results of our experiments suggest that angiogenin could also be effective with respect to other pathological processes, in particular, myocardial infarction, because in this case, the physiological recovery of the functional abilities of the damaged sections of the heart muscle is of prime importance. In our opinion, it would be expedient to study the mechanism of action of this preparation and its effect using other models of tissue damage (stomach ulcer, insult, *etc.*).

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