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Overexpression in *Escherichia coli* and Purification of Human Fibroblast Growth Factor (FGF-2)

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Abstract—Basic fibroblast growth factor (FGF-2) is a member of a large family of structurally related proteins that affect the growth, differentiation, migration, and survival of many cell types. The human *FGF-2* gene (encoding residues 1-155) was synthesized by PCR from 20 oligonucleotides and cloned into plasmid pET-32a. A high expression level (1 g/liter) of a fused protein thioredoxin/FGF-2 was achieved in *Escherichia coli* strain BL21(DE3). The fusion protein was purified from the soluble fraction of cytoplasmic proteins on a Ni-NTA agarose column. After cleavage of the thioredoxin/FGF-2 fusion with recombinant human enteropeptidase light chain, the target protein FGF-2 was purified on a heparin-Sepharose column. The yield of FGF-2 without N- and C-terminal tags and with high activity was 100 mg per liter of cell culture. Mutations C78S and C96S in the amino acid sequence of the protein decreased FGF-2 dimer formation without affecting its solubility and biological activity.

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Basic fibroblast growth factor (FGF-2) is a member of a large family of fibroblast growth factors. There are now known 24 FGF members, and four receptors of these cytokines (FGFR) have been identified [1]. Various cells synthesize FGF-2, which modulates proliferation and differentiation of certain cell types. In addition FGF-2 exhibits potent angiogenic effects *in vivo* and *in vitro*, stimulates growth of smooth muscle cells, wound healing, and tissue regeneration [2]. The wound healing effect makes FGF-2 a potential therapeutic agent of commercial importance. FGF-2 might also play an important role in differentiation and functioning of the nervous system as well as regeneration of eyes and skeleton [3-5].

FGF-2 is a globular protein consisting of a single polypeptide with molecular mass of 18 kDa. FGF-2 molecule contains four cysteine residues, which do not form intramolecular disulfide bonds. Several groups of researchers determined crystal structure of FGF-2 in the presence and in the absence of a heparan sulfate fragment [6, 7]. It was found that FGF-2 consists of 12 antiparallel β-sheets, which form a trigonal pyramid structure. All members of the FGF family bind heparin or heparan sulfates, which influence FGF-2 structure and facilitate formation of dimers and higher order oligomers. Interaction of FGF-2 with heparin protects this protein against heat shock, denaturation in acidic medium, and proteolysis [8]. FGF-2 binds to an extracellular domain of specific receptors (FGFR 1-4) that contain one transmembrane domain. This binding initiates a signal cascade finally resulting in modification in gene expression [9].

FGF-2 has been purified from various bovine organs and tissues including adenoma, brain, hypothalamus, thymus, and kidneys [9]. However, due to very low content it is nearly impossible to obtain significant FGF-2 quantities from animal tissues. Purification of small quantities of FGF-2 is very expensive. The human gene

Abbreviations: ECS, embryonic calf serum; FGF-2, basic fibroblast growth factor; FGFR, receptor of fibroblast growth factor; IPTG, isopropyl-β-D-1-thiogalactopyranoside; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; TB, Terrific Broth (enriched medium); Trx, thioredoxin.

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encoding FGF-2 was cloned and characterized in 1986 [10]. During subsequent decades, several laboratories obtained recombinant FGF-2 from *E. coli* cells [11-15]. Experiments have shown that direct expression of FGF-2 in various bacterial strains yields about 1-10 mg of protein per liter of cell culture. Low yield was partially associated with the fact that FGF-2 is expressed in bacterial systems as inclusion bodies and it undergoes renaturation poorly. A fusion protein glutathione S-transferase/FGF-2 was purified from a bacterial strain with the yield of 20 mg per liter of cell culture; however, the activity of this preparation was significantly lower compared with natural FGF-2 [16]. Substitution of two cysteine residues (C78 and C96) in FGF-2 for serine residues improved protein solubility and its biological activity [15].

In this study, we have developed a new method for preparation of human recombinant FGF-2 in *E. coli*; this results in highly purified and highly active protein preparation of high yield (100 mg per liter of cell culture). The low cost required for this preparation opens possibilities for its use for therapeutic goals.

MATERIALS AND METHODS

Materials. *Escherichia coli* strain BL21(DE3) and a plasmid vector pET-32a were from Novagen (USA); Ni-NTA agarose, kits for isolation and purification of PCR-fragments, for extraction of DNA from gel, and for isolation of plasmid DNA were from Qiagen (USA); restrictases, *Pfu* DNA polymerase, and T4 DNA ligase were from Fermentas (Lithuania); heparin-Sepharose (Fast flow) was from GE Healthcare (USA).

Oligonucleotide design, PCR, and cloning. DNA encoding the amino acid sequence of FGF-2 (513 bp) was synthesized by means of PCR using 20 overlapping oligonucleotides of 35-42 bases in length. For improvement of gene expression in bacterial cells, eukaryotic codons poorly expressed in prokaryotic cells were substituted by corresponding prokaryotic ones. Sites for *Bgl*II restrictases as well as sites for cleavage by enteropeptidase (at the 3′-end) and by *Hind*III (at the 5′-end) were also added to the nucleotide sequence. Two parts of the gene (nucleotides 1-11 and 11-20) were synthesized in separate PCR reactions by means of *Pfu* DNA polymerase. The 50-μl final PCR mixtures contained 50 pmol of each flanking primer and 2.5 pmol of other primers. PCR was carried out by heating of mixtures at 95°C for 5 min followed by subsequent 30 cycles at 95°C for 30 sec, 47°C for 30 sec, and 74°C for 45 sec. PCR products were extracted from the agarose gel and assembly of the full gene from two fragments was achieved by the PCR method using *Pfu* DNA polymerase. The reaction mixture containing 15 ng of each fragment and 50 pmol of each flanking primer was heated at 95°C for 5 min followed by 33 cycles at 95°C for 1 min and 74°C for 1.5 min.

The amplified DNA sequence was cleaved by means of *Bgl*II and *Hind*III restrictases and then cloned into the *Bgl*II/*Hind*III restriction sites of the pET-32a plasmid after the thioredoxin (Trx) gene. The *FGF-2* gene sequence was confirmed by automated sequencing using a ABI Prism-310 gene analyzer. The FGF-2 containing double mutation C78S/C96S was obtained by insertion of nucleotide substitutions into corresponding codons by site-directed mutagenesis using the following primers: 5′-GTCTATCAAAGGTGTGTCTGCTAACCGTTAC-CTG-3′ and 5′-CAGGTAACGGTTAGCAGACACAC-CTTTGATAGAC-3′.

Expression and purification of FGF-2. The *E. coli* strain BL21(DE3) was transformed by the plasmid DNA pET-32a/FGF-2. A single colony of an overnight culture was inoculated into 10 ml of LB medium with ampicillin (100 μg/ml) and grown at 37°C for 17 h. The overnight culture was diluted in TB medium (Terrific Broth; enriched medium) with ampicillin (100 μg/ml) up to light absorption of 0.1 at 550 nm and growth at 37°C for 2-2.5 h up to light absorption of 0.6. After cell induction with 0.02 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside), cultivation was continued at 25°C for 24 h. Cells collected by centrifugation at 4000*g* for 15 min were then disintegrated using a French press in medium containing 50 mM phosphate buffer (pH 8.0), 300 mM NaCl, 10 mM imidazole (lysis buffer). After centrifugation at 100,000*g* for 60 min, the fraction of cytoplasmic proteins was applied onto a Ni-NTA agarose column. Nonspecifically bound proteins were removed by column washing with lysis buffer containing 20 mM imidazole, and then the fused protein was eluted using lysis buffer containing 250 mM imidazole. The purified fusion protein Trx/FGF-2 was dialyzed against 50 mM Tris-HCl buffer (pH 7.8) containing 50 mM NaCl, and then it was cleaved by the catalytic subunit of human recombinant enteropeptidase [17] (during incubation at room temperature for 16 h at the enzyme/substrate ratio of 1 : 75,000). The resulting FGF-2 was separated by affinity chromatography on heparin-Sepharose. The protein was applied onto an affinity column in 50 mM Tris-HCl buffer (pH 7.8) containing 50 mM NaCl. The column was washed with 50 mM phosphate buffer containing 300 mM NaCl; FGF-2 was eluted with the same buffer but with 1.5 M NaCl. The FGF-2 protein preparation was dialyzed against medium containing 50 mM phosphate buffer (pH 7.5) and 150 mM NaCl, sterilized by ultrafiltration, and stored at 4°C. The N-terminal sequence of FGF-2 was determined after SDS-PAGE and trans-blotting onto a PVDF membrane.

Protein concentration during purification was determined by the Bradford method [18]. Concentration of FGF-2 was determined spectrophotometrically at 280 nm using molar absorbance coefficient 14,650 $M^{-1} \cdot cm^{-1}$ estimated from the amino acid sequence on the ExPASy server (www.expasy.org) using the ProtParam program.

SDS-PAGE. Electrophoresis of protein preparations was carried out in 15% SDS-polyacrylamide gel by the method of Laemmli [19]. Proteins were stained using 0.1% solution of Coomassie R-250 in water–methanol–acetic acid (50 : 40 : 10).

Evaluation of biological activity of FGF-2. Biological activity of FGF-2 was tested using a fibroblast NIH3T3 cell line using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test [20]. Initially mouse NIH3T3 fibroblasts were grown in DMEM medium supplemented with 10% embryonic calf serum (ECS) and containing 2 mM glutamine and antibiotics. Cells were seeded onto a 96-well plate (2000 cells per well) in DMEM with 10% ECS. On the next day, cells were washed and cultivated in medium containing 0.5% serum for 18 h, and then FGF-2 was added. After cultivation for 48 h, cells were washed and their number was determined using a CellTiter 96 Aqueous MTS Cell Proliferation Assay (Promega, USA) at 490 nm following the supplier's recommendations.

RESULTS AND DISCUSSION

Expression and purification of FGF-2. For expression of thioredoxin–basic fibroblast growth factor fusion protein (Trx/FGF-2), the *E. coli* BL-21(DE3) strain was transformed by the expressing vector pET-32a/FGF-2. A high level of Trx/FGF-2 expression (about 1 g of liter of cell culture) was achieved after cell induction with 0.02 mM IPTG and subsequent cell growth in TB medium for 24 h at 25°C. Analysis of fractions of soluble and insoluble proteins showed that 50-55% of Trx/FGF-2 existed in soluble form. Trx/FGF-2 was purified from the soluble fraction of cytoplasmic proteins on Ni-NTA agarose. After purification, the fusion was cleaved with the catalytic subunit of recombinant human enteropeptidase. Nonspecific cleavage of Trx/FGF-2 by enteropeptidase was not detected. The molecular mass of the Trx/FGF-2 fusion protein was 34.31 kDa. Cleavage of the fused protein with enteropeptidase yielded FGF-2 (17.25 kDa) and tagged thioredoxin (17.07 kDa). Since the two proteins had almost identical molecular masses, cleavage of the fused protein resulted in appearance of only one band of about 17 kDa on the polyacrylamide gel (Fig. 1, lane *5*). Long-term incubation for 24 h of the fusion protein with enteropeptidase resulted in formation of a protein with molecular mass of 15 kDa (Fig. 1); the latter may be attributed to known nonspecific cleavage of thioredoxin at its C-terminus.

Natural FGF-2 exhibits high affinity towards heparin, and so after the cleavage of the fusion protein Trx/FGF-2 by enteropeptidase final purification of FGF-2 was carried out using a heparin-Sepharose affinity column. Figure 1 shows that purity of resulting preparation of FGF-2 was 95-98%. Yield of purified FGF-2 without N- or C-terminal tags was 100 mg per liter of cell culture.

After transfer of purified FGF-2 onto a PVDF membrane, its N-terminal sequence (MAAGSI) was determined; it corresponds to residues 1-6 of human FGF-2. Mass spectrometry analysis showed only one peak of molecular mass of 17,258 Da (data not shown); this corresponds well to the calculated mass of FGF-2 of 17,253 Da.

Earlier it was demonstrated that substitution of two cysteine residues (C78 and C96) for serines increases solubility of FGF-2 and its biological activity [15]. We introduced these substitutions into the amino acid sequence of FGF-2 by means of site-specific mutagenesis and obtained the mutant variant FGF-2/C78S/C96S using the method developed for the wild-type protein. The yield of FGF-2/C78S/C96S of 105 mg per liter of cell culture was not increased compared with the wild type. SDS-PAGE in the absence and in the presence of β-mercaptoethanol showed that the FGF-2 preparation contains about 5-7% of dimeric molecules, whereas the mutant variant FGF-2/C78S/C96S consists of monomers only (Fig. 2). It is known that in the absence of reducing agents FGF-2 forms oligomers, particularly dimers. Analysis of crystal structure of FGF-2 has shown that dimerization involves cysteine residues C78 and C96, whereas C25 and C92 are inaccessible [21].

It was earlier demonstrated that substitutions of cysteines 78 and 96 for serine improved solubility of recombinant FGF-2 during direct protein expression in *E. coli* and significantly increased yield of this protein [15]. This may be attributed to the fact that in the case of direct expression of FGF-2 in *E. coli* it forms inclusion bodies, whereas the mutant variant FGF-2/C78S/C96S is mainly expressed in

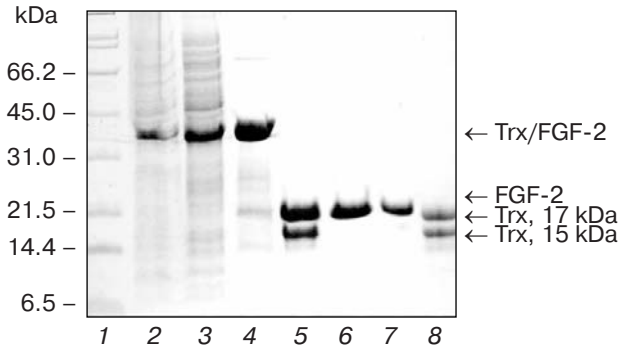

Fig. 1. Expression and purification of human recombinant FGF-2 (SDS-PAGE data). The amount of protein was 10 μg per lane. Lanes: *1*) molecular mass markers; *2*) preparation obtained using an overnight culture; *3*) fraction of cytoplasmic proteins; *4*) fusion protein Trx/FGF-2 preparation after purification on Ni-NTA agarose; *5*) preparation of the fusion protein Trx/FGF-2 after cleavage with enteropeptidase; *6*, *7*) preparation of FGF-2 purified on heparin-Sepharose; *8*) fraction of proteins not bound on heparin-Sepharose.

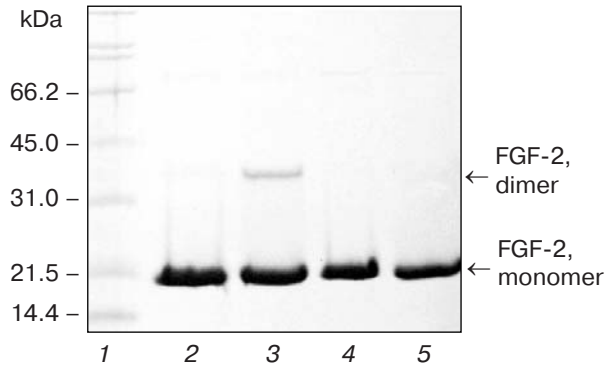

Fig. 2. Presence of dimeric molecules in FGF-2 and FGF-2/C78S/C96S preparations. Each sample containing 15 μg protein was heated at 95°C for 5 min in the presence and in the absence of β-mercaptoethanol and then subjected to electrophoresis in 12% SDS-polyacrylamide gel. Lanes: *1*) molecular mass markers; *2*, *3*) FGF-2 with and without β-mercaptoethanol, respectively; *4*, *5*) FGF-2/C78S/C96S with and without β-mercaptoethanol, respectively.

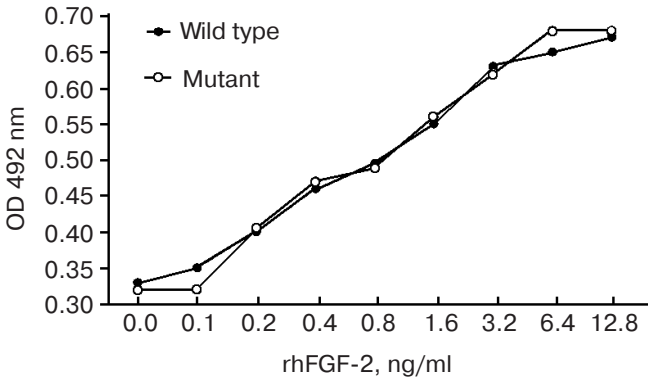

Fig. 3. Comparison of mitogenic activity of FGF-2 and FGF-2/C78S/C96S with respect to NIH3T3 fibroblasts. The number of cells in each well was determined after incubation with various concentrations of FGF-2 for 48 h.

the soluble form. In the case of expression of the fusion proteins (Trx/FGF-2) we did not find any significant quantitative difference in the ratio of soluble and insoluble form of the expressed protein between wild type and the mutant variant Trx/FGF-2/C78S/C96S. This suggests that the carrying protein, thioredoxin, improves solubility of target protein and promotes its folding in cytoplasm.

Biological activity of recombinant FGF-2 and its mutant variant FGF-2/C78S/C96S. Mitogenic activity of the recombinant proteins FGF-2 and FGF-2/C78S/C96S was investigated using the NIH3T3 fibroblast cell line and the MTT test. The two preparations exhibited basically the same (high) activity (Fig. 3). At 3.2 ng/ml concentration, both FGF-2 and FGF-2/C78S/C96S caused a twofold stimulation of growth of NIH3T3 cells, and ED50 (50% effective dose) was 0.3 ng/ml. These results are consistent with mitogenic activity of both native FGF-2 (ED50 of 0.2 ng/ml) and various recombinant preparations (ED50 from 0.3 to 1.2 ng/ml) reported for NIH3T3 cells [15, 22].

Previously it was demonstrated that after substitution of cysteines 78 and 96 for serine, bovine FGF-2 completely retained its mitogenic activity. In addition, its stability in acidic medium increased [23, 24]. Recently a tenfold increase in activity of the human recombinant mutant FGF-2/C78S/C96S compared with wild-type protein was reported [15]. This effect may be associated with the fact that these authors found initial low activity of wild-type FGF-2 (ED50 = 7 ng/ml).

Preparations of FGF-2 obtained in this study exhibited high stability and they retained their total activity after storage at 4°C for at least one year. The mutant variant FGF-2/C78S/C96S was as stable as the wild-type protein (data not shown).

Thus, using our methods for expression and purification of recombinant FGF-2 protein yields highly purified, highly active, and stable preparation. High yield (about 100 mg per liter of cell culture) and low cost of this preparation opens possibilities for its preclinical and clinical trials.

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