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Recent progress in refolding of the C-terminal domain of recombinant human alpha-feto protein

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Abstract

The C-terminal domain of human alpha-fetoprotein (AFP-3D) is a fragment that can be used as a vector for targeted delivery of drugs into tumor cells. A technique of recombinant human AFP-3D refolding in the mobile phase of a gel-filtration medium was developed and shown to produce a 90% yield of the target protein with confirmed functional activity evaluated by the efficiency of AFP-3D-FITC accumulation by MCF-7 cells.

Keywords: alpha-fetoprotein; recombinant proteins; refolding; gel-filtration.

Introduction

Alpha-fetoprotein (AFP) is a plasma protein characteristic of fetal development of all mammals and other animals (Bergstrand and Czar 1956). It nearly disappears from the blood soon after the birth of an animal or a human. However, it can be found in the blood of adults with liver cancer or certain tumors of the reproductive system (Abelev 1971). It was found that cancer cells do not only produce AFP but also take it up, which has been demonstrated for liver cancer, breast cancer, and lung cancer (Villacampa et al. 1984). The presence of AFP receptors on cancer cells was first directly proved in a breast cancer cell culture (Villacampa et al. 1984). It is known that the receptor-binding site is located in the C-terminal domain of the AFP molecule (Mizejewski 2001). Due to these properties, AFP-3D can be used as a vector for targeted delivery of anticancer drugs in tumor cells.

Recombinant AFP-3D can be expressed in *E.coli*, where the protein is accumulated in the insoluble fraction in the form of inclusion bodies. Upon separation, solubilization, and purification of inclusion bodies, the protein must be refolded. The main issues complicating AFP-3D refolding are associated with the presence of a large number of disulphide bonds in the molecule and its highly hydrophobic properties, which result in aggregate formation and decrease the yield of the functional protein. In most cases, dilution technique is used for refolding the protein (Valejo et al. 2004). However, this technique is unlikely to be suitable for hydrophobic proteins and for proteins containing many disulphide bonds. Moreover, the use of large amounts of buffer for refolding consumes large quantities of reagents and requires subsequent protein concentrating. It is recognized that the refolding of proteins containing polyhistidine sequences has a number of advantages (Li et al. 2004). In this case, an appropriate technique is metal chelating chromatography based on complex formation between the protein's polyhistidine sequence and bivalent metal ions, such as Ni^{2+}, Co^{2+}, etc. As a result, protein molecules are efficiently adsorbed on the resin, which, on the one hand, simplifies the purification procedure and, on the other hand, serves for homogenous distribution of the protein within the entire volume of the medium and keeps protein molecules at a certain distance from each other, thus preventing their aggregation. The most common media used in the above procedures are agarose- and silica gel-based matrices carrying nitrilotriacetate/iminodiacetate groups charged with bivalent metal ions. Unfortunately, a common problem associated with this refolding technique is that a certain portion of the protein, depending on the ionic force of solution and on the nature of protein, becomes irreversibly bound to the matrix (Sharapova et al. 2011; Chen and Leong 2009).

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A further alternative technique is refolding by gel-filtration (Chen and Leong 2010). Using an appropriately chosen matrix (resin), it is possible to physically separate the refolding intermediates differing in their hydrodynamic radii and thus to prevent undesirable protein aggregation in the course of renaturation.

Materials and Methods

Expression of recombinant protein in E.coli

The expression system used employed a vector based on the commercial plasmid pET20 containing a fragment of the human AFP gene encoding the C-terminal fragment of the protein (residues 404-595) and seven additional histidine residues (His-tag). The target protein was expressed in *E.coli* BL21(DE3) pUBS cells. Transformation was performed by the standard procedure. Transformed cells were grown at 37°C in LB medium (Amersham, USA) supplemented with 50 µg/ml of ampicillin (Fluka, USA). After the culture reached the OD600 of 0.6, protein synthesis was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) (Helicon, Russia) to the final concentration of 0.5 mM. The culture was incubated with the inductor for 3 h at 37°C, with extensive aeration, and the cells were collected by centrifugation at 3 000g, 4°C, for 10 min. Cell pellets were stored at -20°C.

Isolation, purification, and solubilization of inclusion bodies containing recombinant AFP-3D

The biomass of the producer strain (1 g) was resuspended in 30 ml of phosphate buffered saline (PBS), pH 7.4, containing 2 mM of phenylmethylsulfonylfluoride (Sigma, USA), 2 mM of EDTA, and 0.05 mg/ml of lysozyme (Fluka, Germany) and incubated for 15 min at room temperature. The cell suspension was processed with ultrasound (7×1 min., 270 W, 0.8 sec ON time and 0.2 sec OFF time, 0°C) and centrifuged (10 000 g, 4°C, 10 min). The pellet was resuspended in 30 ml of 10 mM borate buffer, pH 8.4, with 0.1% sodium deoxycholate and incubated for 5-10 min. The suspension was centrifuged at 10 000 g, 4°C, for 10 min. The pellet was washed twice with the same buffer without sodium deoxycholate, and stored at -20°C.

Inclusion bodies (0.2 g) were resuspended in 10 ml of solubilizing buffer (PBS, 8 M urea, 12 mM β-mercaptoethanol, pH 7.4) and incubated with agitation for 1 h at room temperature. The resulting solution was centrifuged at 13 400 g for 10 min and used in further experimental procedures.

AFP-3D purification and refolding by dilution

Solubilized AFP-3D (0.5 mg/ml) was added dropwise to a 20-fold volume excess of renaturing buffer (PBS, 1 mM Glutathione disulfide, 5 mM Glutathione, pH 8.0, 4°C) with extensive agitation, and incubated for 2 days at 4°C without air access. Next, the protein was purified using metal chelating resin, dialyzed against a 1 000-fold volume excess of PBS, pH 8.0, supplemented with glycerin (to 10% v/v), filtered through a 0.22-µm membrane (Millipore, USA), and stored at 4°C.

Refolding of AFP-3D immobilized on Ni-IDA (agarose-based) and on Ni-NTA (silica gel-based)

The renaturing methods differed only in the resins used. Solubilized AFP-3D was applied to the column (10×100 mm, 1 ml/min., 25°C) with Ni-IDA or Ni-NTA resin pre-equilibrated with solubilizing buffer containing 0.01 M imidazole in order to prevent non-specific adsorption. The column was washed with solubilizing buffer until the column effluent adsorption level at 280 nm reached the adsorption level of pure solubilizing buffer. Next, a series of solubilizing/renaturing buffer dilutions to the final concentration of 8, 6, 4, 2, 0 M of urea and 12, 9, 6, 3, 0 mM of β-mercaptoethanol, 1 ml each, were applied to the column in the order of decreasing concentration of denaturing agents. Following that, the column was washed with 5 volumes of renaturing buffer, and AFP-3D was eluted with the same buffer containing 0.3 M imidazole. The AFP-3D obtained was dialyzed against a 1000-fold volume of PBS, pH 8.0, and stored as described above.

Refolding of AFP-3D by gel-filtration using dextran- (Sephadex G25) and agarose- (Superose 12) based matrices.

The renaturing methods only differed in the media used.
Five volumes of renaturing buffer were passed through the column with the medium (10×200 mm, 0.5 ml/min., 25°C). Next, dilutions of solubilizing/renaturing buffers with the final concentrations of 8, 6, 4, 2, 0 M of urea and 12, 9, 6, 3, 0 mM of β-mercaptoethanol, were prepared and applied, 0.5 ml each, to the column, starting from the solution with the lowest concentration of denaturing agents. Solubilized AFP-3D (2 ml of a solution with the concentration of no more than 10 mg/ml) was applied to the prepared column followed by 0.5 ml of solubilizing buffer, and the target protein was then eluted by 3 volumes of renaturing buffer. The eluate was incubated overnight at 4°C, centrifuged at 15 000 g for 15 min, and the supernatant was dialyzed against a 1000-fold volume of PBS, pH 8.0. The resulting protein was filtered through a 0.22-µm membrane and stored at 4°C.

AFP-3D analysis

Protein concentration was determined using a Bicinchoninic Acid Kit (Sigma). The number of free sulfhydryl groups was determined by standard Ellman's test. At all purification stages, the protein was analyzed by Laemmli electrophoresis. The amount of protein was determined by densitometry using the Onedscan program. The homogeneity and purity of renatured AFP-3D were controlled using HPLC (Shimadzu LC-20 Promience, Luna C4 column 2.4×10 mm) with a linear water—acetonitrile gradient (5-60%, 10 min.) containing 0.1% trifluoroacetic acid; the protein was detected at 214 and 280 nm.

Synthesis of FITC-labeled AFP-3D

A 10-fold molar excess of fluorescein isothiocyanate (FITC) (Sigma, USA) in dimethyl sulfoxide was added to an AFP-3D solution in PBS, pH 8.0. The reaction mixture was agitated in the dark for 2 h at room temperature, and then for 12 hours at 4°C. The labeled protein was purified on a Sephadex G25 SF column equilibrated with PBS, pH 7.4. The amount of protein-bound fluorescein was determined at 495 nm, using the molar extinction coefficient $\varepsilon_{495} = 68000\ M^{-1}cm^{-1}$ (Jobbagy and Kiraly 1966). The FITC / protein molar ratio was 2.5 / 1.

Cell culture

Human breast adenocarcinoma MCF-7 cells were maintained in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS; HyClone, USA) and 50 µg/ml of gentamicin (Gibco, USA) in a humidified atmosphere containing 5% CO_2 at 37°C. Peripheral blood lymphocytes of healthy volunteers were isolated by density gradient centrifugation in a Ficoll–Paque gradient and maintained in RPMI 1640 containing 10% of FBS and 50 µg/ml of gentamicin in a humidified atmosphere containing 5% CO_2 at 37°C.

Binding and endocytosis assays

AFP-3D binding to the cell surface and receptor-mediated endocytosis were studied as described previously (Posypanova et al. 2008). Fluorescence was measured using an EPICS XL flow cytometer (Beckman Coulter) equipped with an argon laser ($\lambda_{ex.}$ 488 nm, bandpass 515-520 nm). The mean fluorescence intensity (MFI) was determined in each sample (10^5 cells).

Results and Discussion

AFP-3D isolation

After the 3 hours of IPTG induction, 1 L of culture yielded approximately 70 mg of AFP-3D. The protein formed insoluble aggregates in the form of inclusion bodies. Ultrasound was used to disintegrate the cells; then the inclusion bodies were processed with a solubilizing buffer. The protein content of inclusion bodies was approximately 85%. An electropherogram of the cell culture samples taken prior to and after the induction illustrates AFP-3D production (Figure 1).

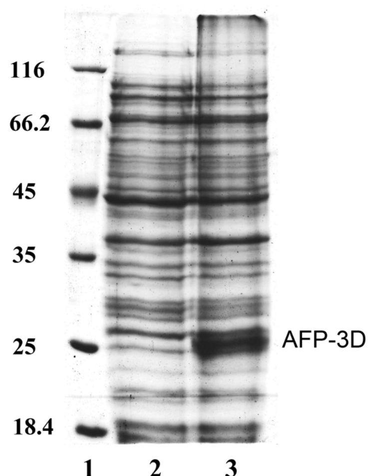

Figure 1: Lysates of transformed *E.coli* cells before induction (2) and after induction (3); molecular mass standards in kDa (1).

Refolding by gel-filtration

Refolding using gel-filtration chromatography is an attractive alternative to refolding by dilution, since protein renaturation and purification can be performed in a single step. In some cases, it was shown that protein misfolding can be avoided by gradually decreasing the denaturing substance content in the mobile phase (Batas and Chaudhuri 1996; Gu et al. 2001). Thus, in this study, we used the technique of gradually reducing the concentration of denaturing substances in the mobile phase of a gel-filtration matrix. The upper part of the column was in the denaturing buffer, while the lower part was in the renaturing one, with a gradient between them. The considerable length of the column and the use of the gradient enabled us to move the protein in the course of chromatography into the buffer containing a Red/Ox pair, as well as to minimize admixtures. In addition, the decreasing gradient of urea also promoted the folding of the target protein and reduced hydrophobic interactions between the protein and the matrix.
According to our data, with this refolding technique, it was possible to minimize AFP-3D losses in the course of renaturation. This positive effect was observed for column loads of up to 20 mg of protein. However, the use of higher protein loads led to formation of large amounts of aggregates and reduced the refolding efficacy (Figure 2).

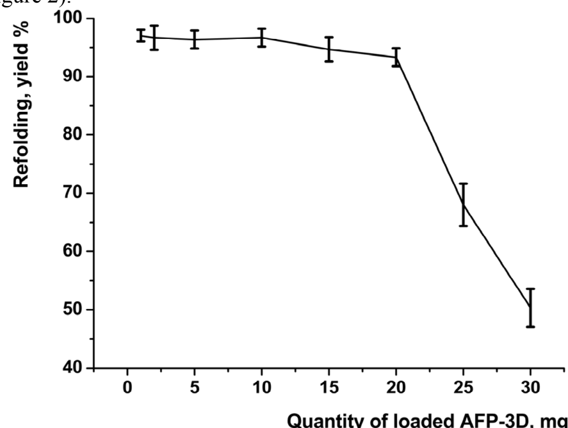

Figure 2: Dependence of the refolded AFP-3D yield on the amount of denatured AFP-3D loaded to the column.

Using the technique presented, we were able to achieve 90% yield of the target protein.

Detecting free sulfhydryl groups

The presence of free sulfhydryl (thiol) groups per protein molecule was determined by the standard method using the Ellman's reagent. No free sulfhydryl groups were detected after refolding.

Analytical chromatography

The AFP-3D homogeneity and purity were controlled using reversed-phase HPLC (Figure 3).

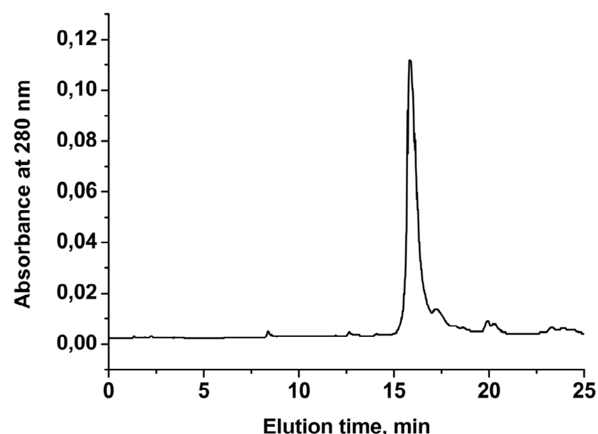

Figure 3: HPLC of the folded AFP-3D. Acetonitrile gradient 5-60% (Symmetry300 C_4, Waters).

The absence of aggregates, also in untargeted fractions, was proved using FPLC with Superose 12 (Figure 4).

Refolding by dilution and by immobilizing on metal chelating resins

As a technique of comparison, refolding by dilution was used. In particular, AFP-3D dissolved in the solubilizing buffer was added dropwise into the renaturing buffer until the protein concentration reached approximately 25 µg/ml and the urea concentration did not exceed 0.4 M. The resulting solution was incubated with intensive

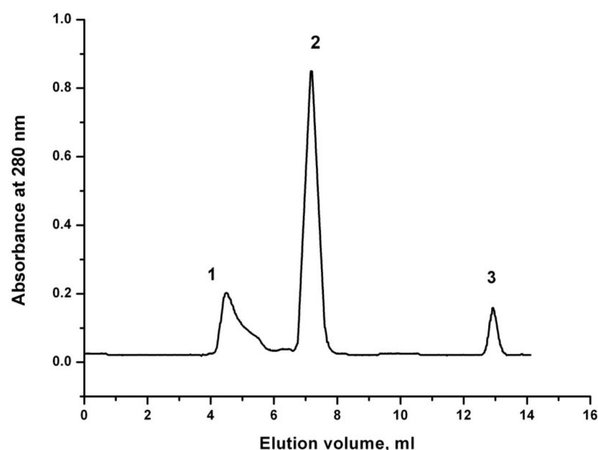

Figure 4: FPLC of AFP-3D folding by gel-filtration (Superose 12). (1) – high molecular weight additives; (2) – AFP-3D; (3) – low molecular weight additives.

agitation at 4°C without air access for 48 h. The renatured protein was purified and concentrated using Ni-NTA Sepharose. The final yield of AFP-3D was 35 mg per 1 L of the culture with a purity of about 96%, which constituted approximately 50% of the initial AFP-3D content of the cell culture. The data obtained agree with the data on human AFP reported previously (Leong and Middelberg 2006).

Refolding by immobilization on metal chelating resins was also used as a comparison technique. Ni-NTA Sepharose and Ni-IDA silica gel were chosen as matrices. A significant shortcoming detected during refolding was a very high level of irreversible AFP-3D adsorption on the media, which reached 40% of the total amount of protein applied. In this case, the irreversibly bound protein could only be eluted in the presence of 1% SDS. The level of irreversible adsorption was the same for both media. The final yield and the purity level for both media were 60% and 98%, respectively.

Functional activity

Functional activity of proteins was evaluated by the efficiency of AFP-3D-FITC accumulation by MCF-7 cells (Figures 5, 6).

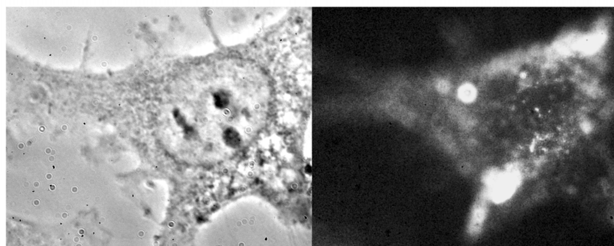

Figure 5: Micrographs of MCF-7 human mammary adenocarcinoma cells after 24 hour incubation with AFP-3D-FITC. The right micrograph was obtained by fluorescent microscopy; the left micrograph was obtained using phase contrast microscopy. Magnification 400×. Microscope Opton IM35 (Germany).

Figure 5 displays micrographs of an MCF-7 cell after 24-hour incubation with AFP-3D-FITC made using fluorescent microscopy. In the right micrograph, bright fluorescent clusters can be clearly seen in the cell cytoplasm, indicating intensive accumulation of AFP-3D-FITC by the cells. The dependence of the MCF-7 cells' fluorescence intensity on the concentration of AFP-3D-FITC folded using different techniques is shown in Figure 6. It was demonstrated

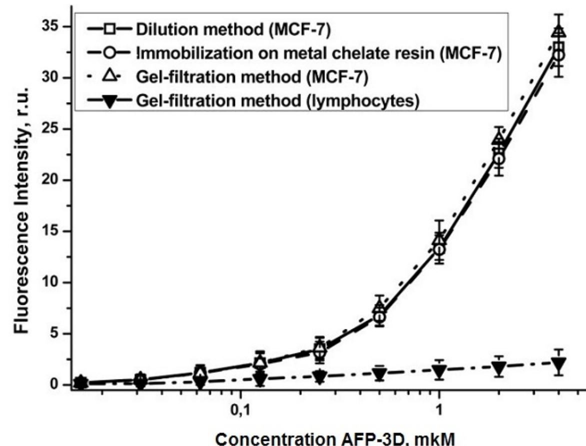

Figure. 6: Intensity of AFP-3D-FITC binding to MCF-7 human mammary adenocarcinoma cells after 1 hour of incubation at +4°C, estimated by MFI of the cells using flow cytometry. R.u. – relative units.

that the efficacy of AFP-3D binding to AFP receptors on the tumor cells surface did not depend on the refolding technique. Pre-incubation of cells with a 10-fold excess of unlabeled human AFP resulted in a significant reduction of AFP-3D-FITC binding to the cells (data not shown), suggesting the involvement of AFP receptors into the endocytosis of AFP-3D.

Conclusions

The study performed has proved the high efficacy of AFP-3D refolding by gel-filtration. In contrast to refolding by dilution or refolding on metal-chelating resins, the use of gel-filtration media minimized the loss of the target protein. The above technique is also characterized with lower buffer amounts required for refolding and a simpler purification procedure. The resulting AFP-3D protein was functionally active and therefore can be used as a targeted vector for selective drug delivery to tumor cells.

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