Journal of the Korean Magnetic Resonance Society **2013**, *17,* 30-39
DOI 10.6564/JKMRS.2013.17.1.030

Expression and Purification of Unstructured Protein, IMUP-1, using Chaperone Co-expression System for NMR Study

Jong-Jae Yi, Jung Ki Yoo, Jin Kyeoung Kim, and Woo Sung Son*

College of pharmacy, CHA University, 120 Haeyong-ro, Pocheon-si, Gyeonggi-do, 487-010, Republic of Korea

Received March 21, 2013; Revised June 4, 2013; Accepted June 10, 2013

Abstract Immortalization-upregulated protein-1 (IMUP-1) genes have been cloned and are known to be involved in SV40-mediated immortalization. IMUP-1 gene is highly expressed in various cancer cell lines and tumors, suggesting the possibility that they might be involved in tumorigenicity. Previously, there were several problems for overexpression of IMUP-1 in bacterial expression systems including low solubility and aggregation due to unstructured property. To investigate the structural properties, it is necessary to obtain lots of pure and soluble proteins. Accordingly, the co-expression systems of bacterial chaperone proteins, GroEL-GroES, were used to increase solubility of IMUP-1. From the analysis of NMR and CD experiment data, it is suggested that the protein adopt typical the random coil properties in solution.

Keywords IMUP-1, Chaperone, Purification, CD, NMR

Introduction

The normal cells lose their ability to divide after a finite number of cell divisions[1, 2]. Although, in this stage called replicative senescence, cell division no longer occur under normal circumstances, additional divisions can be induced, e.g. under the influence of viral proteins. SV40-transformed cells have been intensively studied as a model system for extension of life span[3], and a two-stage model of senescence has been proposed[4]: in mortality stage 1, cells have lost their ability to respond to mitogens. Under the influence of simian virus 40 (SV40) large T-antigen, which interacts with the cell-cycle regulators p53 and retinoblastoma protein (pRb), cells enter a phase of the extended life span (pre-immortalized cells). However, after additional divisions, the cells finally reach the second stage (mortality stage 2), in which the continuing erosion of telomeres (repetitive structures at the linear chromosome ends) does not allow further proliferation, thus resulting in massive cell death[5]. For immortalization, expression of the T-antigen is necessary. Recent studies show that the large T-antigen functions of immortalization and maintaining cells in the immortalized stages can be partially separated[6], thereby drawing a complex picture of the events involved in SV40-mediated immortalization.

Immortalization-up-regulated protein-1 (IMUP-1) was identified as one of the novel transcripts generated by alternative RNA splicing mechanism of a gene located on chromosome 19q13.13 (Figure 1). IMUP-1 protein consists of 106 amino acids and is localized in the nucleus[7]. The function of IMUP-1 is known to be involved in SV40-mediated immortalization[7]. Also, IMUP-1 is up-regulated in SV40-immortalized cells, but not yet in SV40-transformed and mortal cells. Although IMUP-1 gene is expressed in some normal tissues, they are more frequently and more highly expressed in cancer cell

* Address correspondence to: **Woo Sung Son,** College of Pharmacy, CHA University, 120 Haeryong-ro, Pocheon-si, Gyeonggi-do, 487-010, Republic of Korea, Tel: +82-31-850-9398; Fax: +82-31-850-9398; E-mail: wson@cha.ac.kr

lines such as lung, colon, and ovarian carcinoma cell lines[8]. It has been reported that IMUP-1 expression in ovarian cancer cell lines and ovarian epithelial tumors is significantly higher than in normal ovarian cell lines and tissues[9]. This suggests that IMUP-1 might be implicated in tumorigenesis. However, the biological mechanism of IMUP-1 is not known until recently. To understand the functional role of the IMUP-1 in the molecular level, it is necessary to obtain the structural properties. We have investigated the secondary structure of IMUP-1 using circular dichroism (CD) and nuclear magnetic resonance (NMR). For these measurements, IMUP-1 was co-expressed chaperone, and highly purified protein was obtained using liquid-chromatography.

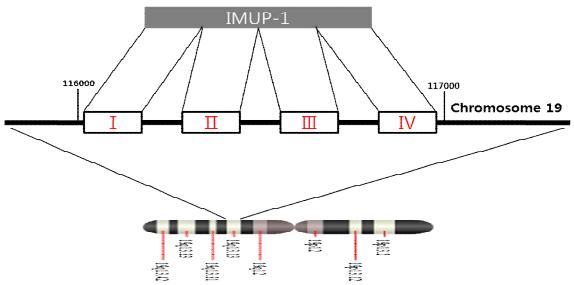

Figure 1. IMUP-1 by RNA alternative splicing of a gene located on chromosome 19.

The mass production of recombinant protein in *Escherichia coli* is one of the major efforts in biotechnology. And it is very important to fold in their biologically active conformations within the bacterial cell because *in vivo* protein folding is quite different phenomenon compared with in bacterial cell, complicated by macromolecular crowding in cytosol. The mechanism of protein folding in life remains one of the most compelling problems in molecular biology[10]. The *in vivo* folding mechanisms are affected by a number of factors, such as physico-chemical conditions of the cellular environment and transient interactions with other co-translated proteins, not present in the simplified *in vitro*. In the complex medium of the cell, the physical conditions of temperature, pH, etc., are restricted, and the concentration of macromolecules is high creating a dynamically changing environment for newly synthesized proteins[11]. The chaperones are a class of proteins thought to facilitate protein folding in this environment[12]. As unfolded proteins contain many exposed hydrophobic residues than the proteins in its native state, they are much more susceptible to aggregation. The Chaperones are thought to prevent newly synthesized proteins from misfolding and aggregation, impeding undesired hydrophobic interactions, and allowing alternative folding pathways[13]. They bind to the exposed hydrophobic regions of nonnative proteins, hindering aggregation[14]. Therefore, through regulated cycles of peptide binding and release, chaperones facilitate the acquisition of the active conformation of the proteins. The most extensively studied chaperones are the chaperonin, GroEL and GroES, from *E. coli*[15]. The structure of GroEL[16] and GroES[17] are known and also that of the GroEL-GroES complex formed in the presence of ADP[18]. The mechanism of protein folding with chaperone is as follows in Figure 2[19]. First of all, the GroEL protein participates in an asymmetric complex, the cis configuration, with one heptameric ring of GroES and 14 ADP's bind to GroEL subunit each one. GroEL binds to a denatured polypeptide in its central cavity and releases all 14 ADP's and the GroES molecule. As next step, the GroEL binds to 14 ATP's and then the binding of ATP reduces affinity of GroEL for the substrate protein. The interaction between GroEL and the substrate weaken thereby allowing the GroES protein to bind to the opposite face of GroEL, trans configuration. In third process, all 14 ATP's are hydrolyzed simultaneously thus releasing the bound polypeptide from the GroEL cavity. This release allows the polypeptide, which is probably in the molten globule state, to fold in a protected microenvironment and out of contact with other denatured proteins with which it would otherwise aggregate. Finally, if the polypeptide has completely folded into its native conformation, it is completely released from GroEL. If the polypeptide is only partially folded, it will rebind to the chaperonin, thus perpetuating the cycle until folding is complete. GroES binds but not hydrolyze ATP. In addition to

facilitating the binding of ATP, GroES coordinates the 14 ATP's simultaneous hydrolysis and prevents the escape of a partially folded polypeptide from the GroEL cavity. The cycle is mechanistically coupled so that the reaction is self-perpetuating until protein folding has finished. Eventually, the folded protein will have lost its affinity for GroEL.

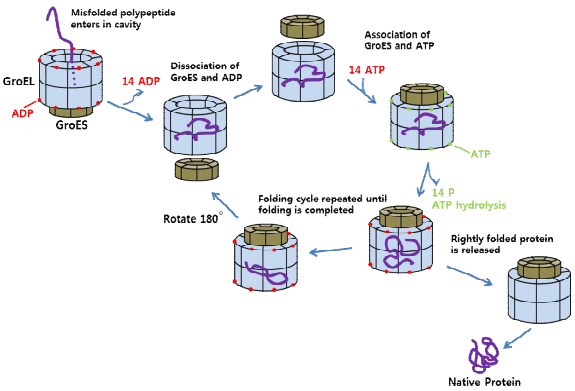

Figure 2. Mechanism of chaperonin assisted protein folding.

Experimental Methods

Materials- Oligonucleotides that are primer for the polymerase chain reaction (PCR) were synthesized by Bioneer, Inc. (Daejeon, Korea). PCR premix kits were purchased from iNtRON BIOTECHNOLOGY, Inc. (Gyeonggi, Korea). DNA restriction enzymes, BamH1and Xho1, were from New England Biolabs (NEB). T4 DNA ligase and the Chaperone Competent Cell BL21 Series (pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16) were purchased from TaKaRa Bio, Inc. (Shiga, Japan). The pGEX-4T-1 plasmid was from GE Healthcare Life Sciences (Pittsburgh, USA). The DH5α, BL21, BL21 (DE3) pLysS, C41 (DE3) and C43 (DE3) *E. coli* strains were kindly offered from professor Bong-Jin Lee (College of Pharmacy, Seoul National University). Ampicillin was from Invitrogen (California, USA). The kits for plasmid extraction and DNA purification were purchased from GeneAll (Seoul, Korea). Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Biosesang (Gyeonggi, Korea). Human alpha thrombin was from Enzyme Research Laboratories (Swansea, UK). Tetracycline, chloramphenicol, L-Arabinose and other reagents to make buffer except isotopes were from Sigma Chemical Company (St. Louis, USA). Isotope such as ^{15}N source (^{15}N-NH_4Cl) was purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA). Glutathione S-transferase (GST) affinity column (5 ml, GSTrap HP) and cation exchange column (5ml, HiTrap SP-FF) from GE Healthcare Life Sciences (Pittsburgh, USA) were used for the purification of protein. Polymerase chain reaction was carried out by T100-Thermal cycler, Bio-Rad (California, USA). The sonicator, VC- 650, for cell lysis was from SONICS&MATERIALS, Inc. (Connecticut, USA). The centrifugation was performed by Beckman Instruments Inc. (California, USA). FPLC, AKTA prime plus, from GE Healthcare Life Sciences (Pittsburgh, USA) was operated for purification of protein. Amicon Ultra-15 (3,000 cut) from EMD Millipore (Massachusetts, USA) was used to exchange buffer and to concentrate the final sample. All CD spectra were measured with JASCO J-715 spectropolarimeter (Victoria, British Columbia). NMR tubes were prepared from Shigemi Inc. (Tokyo, Japan). Bruker DRX-600 MHz NMR spectrometer from Bruker NMR Instruments (Massachusetts, USA) was used to obtain the HSQC spectrum.

Construct cloning- The IMUP-1 gene encoding residues 1-106 was amplified by polymerase chain reaction (PCR) using template that was cloned into pET-28a. Then the PCR product was cloned into pGEX-4T-1 expression vector by using standard protocols. The restriction enzymes, BamH1 and Xho1, were used for insertion of PCR products into plasmid. This vector contains thrombin cleavage site and the glutathione S-transferase (GST) tag for solubility and purification. Sub-cloning work was performed in *E. coli*, DH5α strain. The plasmid was extracted by using Plasmid SV mini prep. kit (GeneAll). This plasmid was transformed into *E. coli* strain BL21 (DE3) pLysS, C41 (DE3) and C43

(DE3) for protein expression. DNA sequencing was performed by Bioneer, Inc. (Daejeon, Korea). Analyzed DNA sequence was identical with original gene sequence.

Optimization of expression and solubility- The overproducing *E. coli* strain BL21 (DE3) pLysS, C41 (DE3) and C43 (DE3) containing the specific plasmid of IMUP-1 with resistance to ampicillin was used for the expression and solubility test. Cell stock stored at -80℃ was cultivated on agar plate for 10 h with ampicillin (100 ㎍/ml final conc.) at 37℃. To over-express, the colony was inoculated into 6 ml LB medium containing 100 ㎍/ml ampicillin. After reaching the OD of cell growth about 0.6 at 37℃, the expression of IMUP-1 was induced for 8 h by adding Isopropyl β-D-thiogalactopyranoside (IPTG) to final concentration of 1 mM. The grown cells were harvested by centrifugation at 14,000 rpm for 3 min. The expression of fusion protein was identified by 15% Glycine SDS-PAGE. As the next step, we carried out culture again using the same methods of the expression test to confirm solubility. To check best condition of soluble expression, cells were induced by adding IPTG to final concentration of 0.1, 0.5, 1.0 and 2.0 mM at 15℃ and 37℃. After 8 h induction, cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 lysis buffer. The cells were lysed by sonication with five cycles (3 s on and 5 s off of pulse) for 2 minutes, amplitude 30% at 650 W on ice. The lysate was centrifuged at 14,000 rpm for 20 minutes to gain the soluble supernatant. To identify solubility, separated pellet and supernatant were loaded 15% Glycine SDS-PAGE.

Co-expression with the Chaperone- Using the chaperone co-expression, BL21 were doubly transformed with the expression plasmid for target protein and the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16, respectively. After co-expression test, we decided the best chaperone that is the pGro7 plasmid. It contains the expression system for GroEL-GroES. Co-expression of IMUP-1 and GroEL-GroES were carried out in LB and M9 minimal medium containing 100 ㎍/ml ampicillin at 37℃ until the cells reached the mid-log phase. In order to induce expression of chaperone, GroEL-GroES, 0.5 mg/ml L-Arabinose was added to 6 ml culture medium when the cells reached the optical density (OD) of cell at 600 about 0.4 and then they were incubated at 15℃ until OD_{600} about 0.6. For soluble expression, low temperature was required. Subsequently, the target protein expression was induced by 1.0 mM IPTG at 15℃ for 12 h in LB and for 12-20 h in M9 minimal medium. The cells were harvested by centrifugation with 8,000 rpm at 4℃ for 10 min. The pellet was lysed by sonication with five cycles (3 s on and 5 s off of pulse) for 2 minutes, amplitude 30% at 650 W on ice. The lysate was centrifuged at 14,000 rpm for 20 minutes to gain the soluble supernatant. To identify solubility, separated pellet and supernatant were loaded 15% Glycine SDS-PAGE. The expression and solubility level was analyzed by 15% Glycine SDS-PAGE.

Over-expression and purification- Cultivate of doubly transformed BL21 with the expression plasmid for target protein and the plasmid pGro7 was scaled up to 1.0 liter medium. In this stage, culture condition was equal to previous experiment (2.2.3. co-expression with chaperone). The harvested pellet was resuspended in 50 ml lysis buffer (50 mM Tris-HCl, 150mM NaCl, pH 7.5). After cell resuspension, it was lysed by sonication and operated with five cycles (3 s on and 5 s off of pulse) for duration time 10 min, amplitude 45% on ice. Cell debris was isolated by centrifugation at 14,000 rpm, 4℃ for 40 min and supernatant was filtered by syringe filter (0.45 ㎛). GST affinity column (5 ml, GSTrap HP, GE Healthcare) was used as a first step of purification. GST column was equilibrated by using the lysis buffer. Then separated supernatant from cell debris was loaded to column (1 ml/min flow rate). The loaded column was washed with same buffer (50 mM Tris-HCl, 150mM NaCl, pH 7.5). The fusion protein bound to the resin was eluted by using linear gradient to 100% elution buffer (50 mM

Tris-HCl, 150 mM NaCl, 30 mM Glutathione, pH 7.5). Subsequently, fractions containing IMUP-1 were pooled and concentrated by Amicon Ultra-15 (3,000 cut, Millipore) to cleavage GST tag and change buffer condition (50 mM Tris-HCl, 200 mM NaCl, pH 7.5). GST and IMUP-1 were cut by the human alpha thrombin (Enzyme Research Laboratories) for 6 h in cleavage buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5) at 20℃. Cut proteins were separated by GST affinity chromatography. Detached protein IMUP-1 from GST tag was dialyzed to desalt a 10 kDa molecular weight cutoff (MWCO) tubing for 5 h in dialysis buffer (50 mM Tris-HCl, 10 mM NaCl, pH 7.5) at room temperature. After desalting, IMUP-1 was loaded to equilibrated cation-exchange column (5ml, HiTrap SP-FF, GE Healthcare) with binding buffer (50 mM Tris-HCl, 10 mM NaCl, pH 7.5) and eluted by using gradient to 100% elution buffer (50 mM Tris-HCl, 1 M NaCl, pH 7.5). As the final step, the eluted protein was changed buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.0) and concentrated by using Amicon Ultra-15 (3,000 cut, Millipore) for CD analysis. For NMR measurements, N-NH_4Cl was used as ^{15}N source in M9 minimal medium. The experimental step and condition to prepare NMR sample was equal to previous methods except medium and final buffer. ^{15}N-labeled IMUP-1 was prepared with 50 mM Sodium phosphate buffer, 10% DO, pH 6.0. The purity of protein at each step was confirmed by 15% Glycine SDS-PAGE.

Structural bioinformatics- The secondary structure prediction was performed by GOR Ⅳ[20]. The prediction of intrinsically unstructured proteins (IUPs) was carried out by IUPred[21].

Circular dichroism (CD)- CD spectra were recorded on a JASCO J-715 spectropolarimeter (Victoria, British Columbia) equipped with a thermoelectric temperature controller. Far-UV CD spectra were monitored from 250 nm to 190nm using the protein concentration of 50uM in cuvette with a path length of 0.1 cm, a response time of 1s, and a scan speed of 20 nm/min at 25℃. Three scans were added and averaged, followed by subtraction of the CD signal of the solvent. For pH titration experiment, the pH of samples were prepared 4.0, 5.0 (50 mM sodium citrate, 100 mM NaCl), 6.0, 7.0 (50mM sodium phosphate, 100 mM NaCl) and 8.0 (50mM Tri-HCl, 100 mM NaCl), respectively.

Nuclear magnetic resonance (NMR)- NMR experiments were conducted at 303K using Bruker DRX-600 MHz spectrometers. ^{15}N-labeled IMUP-1 for NMR measurement was grown in M9 midium containing ^{15}N-NH_4Cl and concentrated up to 1 mM with 50 mM sodium phosphate, pH 6.0 (90% H_2O / 10% D_2O for locking). NMR spectrum was processed using NMRPipe[22], NMRView[23]software.

Results

Optimizing of expression and solubility- Expression and solubility test were performed by using BL21 (DE3) pLysS, C41 (DE3) and C43 (DE3) competent cells. As a first work, expression test was carried out with general methods. IMUP-1 was expressed in all competent cells. The expression of fusion protein was identified by 15% Glycine SDS-PAGE. The results of expression test are shown in Figure 3. Subsequently, solubility test of target protein was carried out with various conditions. To find the best

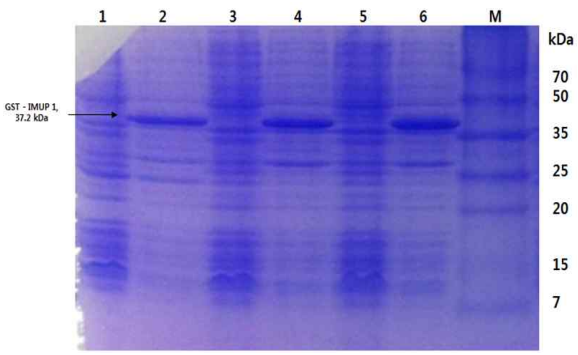

Figure 3. Expression of IMUP-1. The GST-IMUP-1 fusion protein (37.2 kDa) was expressed in BL21 (DE3) pLysS, C41 (DE3) and C43 (DE3) strains. Lane 1, 3 and 5: the cells before induction, Lane 2, 4 and 6: the cells after induction (lane 1, 2: BL21 (DE3) pLysS, lane 3, 4: C41 (DE3), lane 5, 6: C43 (DE3)).

condition of soluble expression, cells were induced by 0.1, 0.5, 1.0 and 2.0 mM IPTG at 15℃and 37℃. Induced cell was harvested and lysed to identify solubility. We confirmed behavior of fusion protein through 15% Glycine SDS-PAGE. Most of the expressed IMUP-1 was found as insoluble form in the pellet, regardless of the temperature and concentration of IPTG (Figure 4). And the amount of soluble protein was almost same in the various cell lysis buffer (The results are not shown).

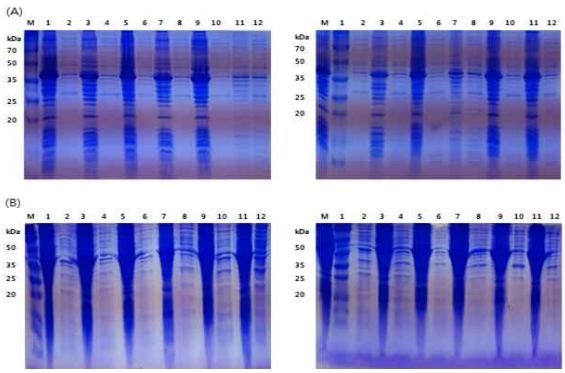

Figure 4. Solubility of IMUP-1. The odd numbers indicate pellet and even numbers indicate supernatant. Lane 1, 2, 9, 10, 17 and 18: induction by 0.1 mM IPTG. Lane 3, 4, 11, 12, 19 and 20: induction by 0.5 mM IPTG. Lane 5, 6, 15, 16, 23 and 24: induction by 1.0 mM IPTG. Lane 7, 8, 15, 16, 23 and 24: induction by 2.0 mM IPTG (lane 1-8: BL21 (DE3) pLysS, lane 9-16: C41 (DE3), lane 17-24: C43 (DE3) both A and B).

Co-expression with the GroEL-GroES- In order to obtain more of the over-expressed IMUP-1 in the soluble fraction, we tried to co-express the protein with the prokaryotic chaperone proteins, GroEL-GroES, after test the five types of plasmid in Figure 5. The chaperones were composed of five types of plasmid, respectively (Table 1). GroEL and GroES are well-known prokaryotic molecular chaperones, which facilitate folding of proteins[24]. Nishihara e.t al. reported that co-expression of GroEL-GroES were effective to improve the solubility of bacterially over-expressed recombinant proteins[25]. Here, we used previously described the plasmid pGro7 that over-expressed GroEL-GroES under the control of the araB promoter inducible with

Table 1. Chaperone team coded on each plasmid.

No.	Plasmid	Chaperone	Promoter	Resistant Marker	Inducer(final conc.)
1	pG-KJE8	dnaK-dnaJ-grpE	*araB*	Cm	L-Arabinose (0.5 mg/ml)
		groES-groEL	*Pzt-1*		Tetracyclin (1 - 5 ng/ml)
2	pGro7	groES-groEL	*araB*	Cm	L-Arabinose (0.5 mg/ml)
3	pKJE7	dnaK-dnaJ-grpE	*araB*	Cm	L-Arabinose (0.5 mg/ml)
4	pG-Tf2	groES-groEL-tig	*Pzt-1*	Cm	Tetracyclin (1 - 5 ng/ml)
5	pTf16	tig	*araB*	Cm	L-Arabinose (0.5 mg/ml)

L-Arabinose. The chaperone plasmid pGro7 carried a pACYC origin of replication plus a chloramphenicol resistance which allows their use with most common ColE1-type plasmid having ampicillin resistance gene as a marker. As chaperone gene is located at the downstream of araB promoter, separate expression of chaperone and target proteins can be accomplished if the target gene is placed under the control of a different promoter including lac. The pGro7 plasmid is not available for the combination use with a plasmid containing chloramphenicol resistance gene. *E. coli* BL21 was doubly transformed with pGEX-4T-1 containing gene that is encoded target protein and pGro7, and we compared the solubility of the over-expressed IMUP-1 obtained with and without pGro7. The solubility of IMUP-1 was dramatically improved with the co-expressed GroEL-GroES (Figure 5). The soluble portion of the totally expressed recombinant IMUP-1 was about 60% with the chaperone, while almost nothing without them. Cultivate was scaled up to 1.0 liter and IMUP-1 was over-expressed with chaperone using

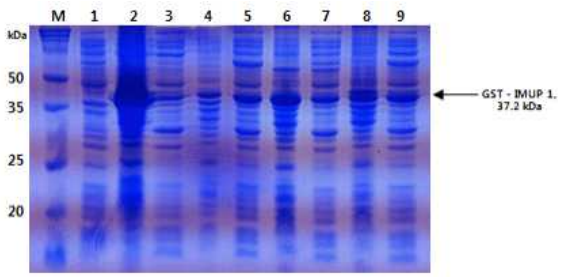

Figure 5. The GST fusion IMUP-1 was co-expressed with chaperone. Lane 1: before induction, Lane 2, 3: dnaK-dnaJ-grpE, Lane 4, 5: groES-groEL, Lane 6, 7: groES-groEL-tig, Lane 8, 9: dnaK-dnaJ-grpE/ groES-groEL (lane 2, 4, 6 and 8: pellet / lane 3, 5, 7 and 9: supernatant).

optimized methods (In this step, cells were grown in M9 minimal medium containing ^{15}N-NH_4Cl as the sole nitrogen source).

Purification of GST-IMUP-1 fusion protein- The cells were harvested after over-expression and lysed by sonication. The cell debris was removed by centrifugation and the soluble and insoluble fraction were collected and analyzed by 15% SDS-PAGE. After clarification, the IMUP-1 was purified by using the GST affinity column. It was loaded with flow rate of 1 ml/min and was eluted using gradient to 30mM Glutathione. The IMUP-1 bound to the column was nearly eluted with about 6-10 mM Glutathione in Figure 6. The fractions including IMUP-1 were pooled and concentrated to cleavage GST tag and change buffer condition. GST and IMUP-1 were cut by the human alpha thrombin for 6 h in cleavage buffer at 20℃ (Figure 7a). Digested proteins were separated by GST affinity chromatography in Figure 7 b. Separated IMUP-1 from GST tag was dialyzed to desalt using 10 kDa molecular weight cutoff tubing. After desalting, IMUP-1 was concentrated in Figure 7 c and loaded onto cation-exchange column and eluted using gradient to 1 M NaCl. IMUP-1 was eluted with 750 mM NaCl (Figure 8). As a final step, the eluted protein was changed buffer and concentrated for measurement of CD and NMR.

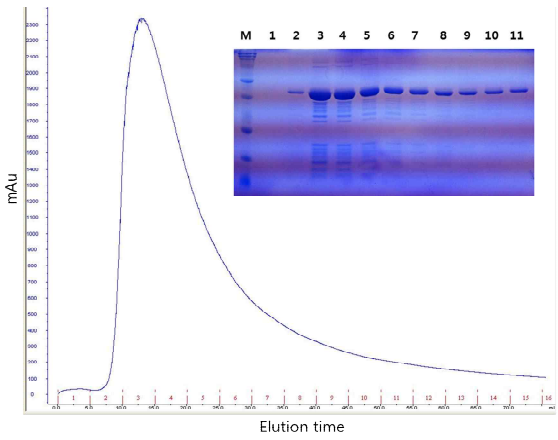

Figure 6. FPLC chromatogram of eluted IMUP-1 using the GST column. The flow rate was 1 ml/min and peak was detected at 280 nm UV.

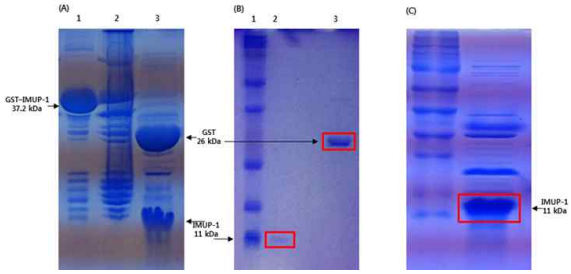

Figure 7. The fusion protein GST-IMUP-1 was cut by the human alpha thrombin and was separated using GST column. (A) Lane 1: before thrombin cleavage, Lane 2: aggregated protein during thrombin cutting, Lane 3: after thrombin cleavage. (B) Lane 1: separated IMUP-1, Lane 2: GST tag. (C) IMUP-1 was concentrated after separation.

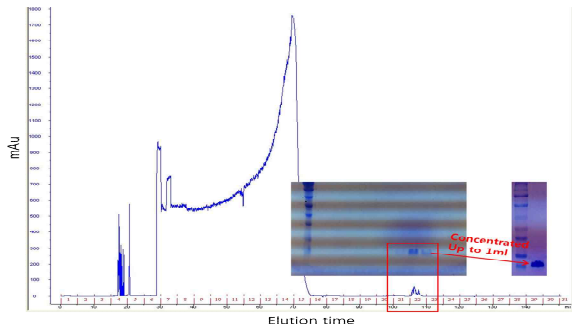

Figure 8. The concentrated IMUP-1 was purified using the cation-exchange chromatography. This chromatogram shows that IMUP-1 was eluted with 750 mM NaCl.

Structural bioinformatics- Physicochemical information of IMUP-1 were obtained using bioinformatics tools. From the ProtParam prediction, theoretical pI value was 9.73 [26]. Secondary structure prediction was made by GOR Ⅳ[20]. Secondary structure of IMUP-1 was predicted as 15.09% alpha helix, 5.66% extended strand and 79.25% random coil using GOR method (Figure 9). Disordered tendency was predicted by IUPred[21]. Disorder prediction score is described in Figure 10. This score can be between 0 and 1 and scores above 0.5 indicate disorder.

Circular dichroism (CD)- In order to analyze a secondary structure of IMUP-1, the far-UV CD

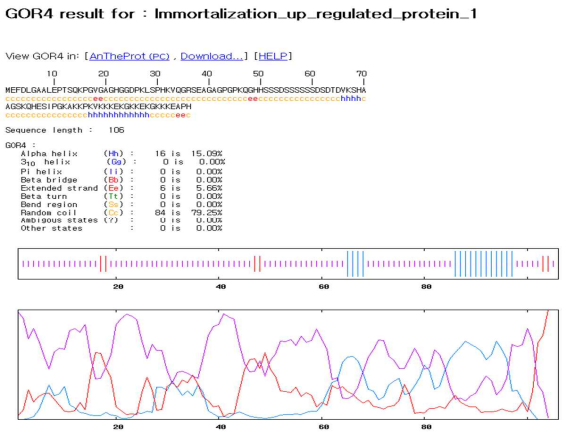

Figure 9. Result of GOR Ⅳ bioinformatics tool.

Figure 10. Disorder prediction of IMUP-1 by IUPred. For prediction types "local" and "global" disorder there is a line corresponding to each residue, specifying its sequential number, residues type, and its score. This score can be between 0 and 1. Scores above 0.5 indicate disorder.

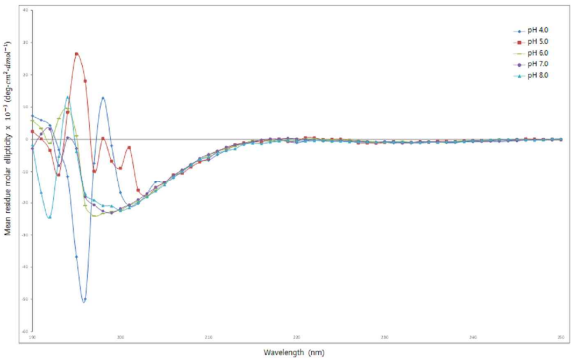

Figure 11. CD spectra of IMUP-1 with Far-UV pH titration. pH 4.0, 5.0 (50 mM sodium citrate, 100 mM NaCl), pH 6.0, 7.0 (50 mM sodium phosphate, 100 mM NaCl), pH 8.0 (50 mM Tris-HCl, 100 mM NaCl).

spectra were measured. The spectrum was detected from 250 nm to 190 nm. The pH titration was performed to check property of protein at each pH. As expected, these CD spectra show almost random coil behavior of IMUP-1 in Figure 11.

Nuclear magnetic resonance (NMR)- The spectrum of IMUP-1 was analyzed 2D N-HSQC by using 600 MHz NMR spectrometer in solution state. ^{15}N-labeled IMUP-1 was prepared at concentration of 1 mM in 50 mM sodium phosphate, pH 6.0 (90% H_2O, 10% D_2O). IMUP-1 is composed 106 amino acids, but it was observed only 31 peaks (Figure 12).

Discussion

IMUP-1 is highly expressed in various cancer cell lines and tumors, suggesting that they might be involved in tumorigenecity[8,9]. The biological mechanism of IMUP-1 is not yet known. And three-dimensional structure is crucial to understand role and mechanism of target protein. To elucidate three-dimensional structure of target protein, it is essential to obtain lots of highly purified protein samples. We developed cloning, expression and purification methods for mass production of protein. Also, structural properties of protein were monitored using computational prediction, CD, and NMR for optimizing NMR sample condition.

We could get amounts of the protein, but their solubility was low. Generally, unstructured protein did not well expressed in bacterial cell. Therefore, unstructured property of IMUP-1 protein could lead to low solubility *in vitro*. Usually, aggregated protein is refolded by using including urea and guanidine-HCl. In this case, we used chaperone system for proper folding instead of refolding method for purification because the main reason of aggregation of IMUP-1 protein could be unstructured property.

We assumed that the chaperone proteins could help appropriate folding of IMUP-1. Five types of chaperone (Table 1) were used to find best co-expression system. We could confirm that solubility of IMUP-1 was dramatically improved

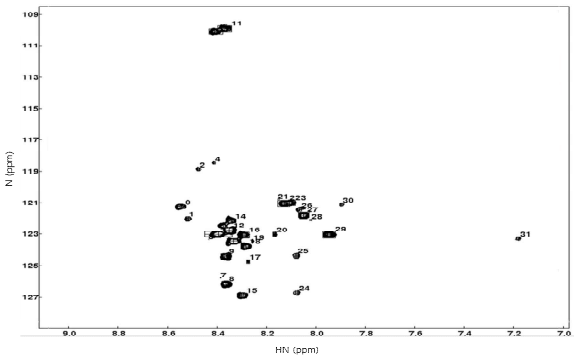

Figure 12. 2D-[^{1}H-^{15}N]-TROSY HSQC spectrum of the unstuctured protein IMUP-1. This NMR spectrum was acquired at 303K on a Bruker DRX 600 spectrometer.

with co-expressed GroEL-GroES. The soluble portion of the totally expressed IMUP-1 was increased about 60% with the chaperone. As the next step, IMUP-1 was purified from impurities using GST affinity chromatography and cation-exchange chromatography. To analyze a secondary structure of target protein, CD experiment was performed at each pH 4.0 - 8.0.

NMR measurement was carried out to assign amino acid sequence of IMUP-1. But only 31 peaks were observed among the entire 106 amino acids in the spectrum. The CD and NMR spectrum showed that IMUP-1 adopts the random coil in solution. To investigate structure and function of IMUP-1, it is necessary to get properly folded structure of protein. And, it is expected that binding with DNA/RNA or other proteins could lead to correct folding for structure determination.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2012R1A1A1043288) and supported by the Industry Academic Cooperation Foundation funded by the CHA University [CHAACF-2012-0041]. This study also supported by the Lab. For Structure Based Drug Discovery, College of Pharmacy, Seoul National University.

References

1. Hayflick, L., Moorhead, P. S. Cell Res. **25**, 585. (1961).
2. Hayflick, L. Cell Res. **37**, 614. (1965).
3. Jha, K. K., Banga, S., Palejwala, V., Ozer, H. L. Exp. Cell Res. **245**, 1. (1998).
4. Wright, W. E., Pereira-Smith, O. M., Shay, J. W. Mol. Ell. Biol. **9**, 3088. (1989).
5. Reddel, R. R. Ann. New York Acad. Sci. **854**, 8. (1998).
6. Powell, A. J., Darmon, A. J., Gonos, E. S., Lan, E. W., Peden, K. W., Jat, P. S. Oncogene. **18**, 7343. (1999).
7. J. K. Kim, R. Roland, I. Yoshiko, K. Seishi. Gene. **257**, 327. (2000).
8. Z. Y. Ryoo, B. K. Jung, S. R. Lee, M. O. Kim, S. H. Kim, H. J. Kim, J. Y. Ahn, T. H. Lee, Y. H. Cho, J. H. Park, J. K. Kim. Biochem Biophys Res Commun. **349**, 995. (2006).
9. J. K. Kim, H. J. An, N. K. Kim, J. Y. Ahn, K. S. Kim, Y. J. Kang, J. J. Ko, D. Oh, C. Lee, S. J. Kim, K. Y. Cha. Anticancer Res. **23**, 4709. (2003).
10. Cortazzo, P., Cervenansky, C., Marin, M., Reiss, C., Ehrlich, R., & Deana, A. Biochemical and Biophysical Research Communications. **293**, 537. (2002).
11. Johnson, J. L., & Craig, E. A. Cell. **90**,201. (1997)..
12. Horwich, A. L., Low, K. B., Fenton, W. A., Hirshfield, I. N., & Furtak, K Cell. **74**, 909. (1993).

13. Hartl, F. U. Nature. **381**, 571. (1996).
14. Hartl, F. U., & hayer-Hartl, M. Science. **295**, 1852. (2002).
15. Fenton, W. A., & Horwich, A. L. Science. **6**, 743. (1997).
16. Brain, K., Otwinowdki, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., et al. Nature. **371**, 578. (1994).
17. Hunt, J. F., Weaver, A. J., Landry, S. J., Gierash, L., & Deisenhofer, J. Nature. **379**, 37. (1996).
18. Xu, Z., Horwich, A. L., & Sigler, P. B. Nature. **388**, 741. (1997).
19. Jonathan, S. W., Hays, S. R., Wayne, A. F., Joseph, M. B., Arthur, L. H. Cell Press. **84**, 481. (1996).
20. Garnier, J., Garnier, J. F., Robson, B., R. F. Doolittle, Editor. **266**, 540. (1996).
21. Dosztanyi, Z., Csizmok, V., Tompa, P. and Simon, I. J. Mol. Biol. **347,** 827. (2005).
22. Delaglio, F., et al., J. Biomol. NMR. **6(3)**, 277. (1995).
23. Johnson, B. A. and R. A. Blevins, J. Biomol. Nmr. **4(5)**, 603. (1994).
24. P. Goloubinoff, A. A. Gatenby, and G. H. Lorimer, Nature. **337,** 44. (1989).
25. Nishihara, K., Kanemori, M., Yanagi, H., & Yura, Y. Applied and Environmental Microbiology. **66,** 884. (2000).
26. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, Marc R., Appel, Ron. D., Bairoch, A., J. M. Walker, Editor. Humana Press. **52**,571.(2005).