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Molecular Cloning and Overexpression of WAP Domain of Anosmin-1 (a-WAP) in *Escherichia coli*

Srinivas Jayanthi[1], Beatrice Kachel[1], Jacqueline Morris[1], Igor Prudovsky[2] and Thallapuranam K. Suresh Kumar[1]
[1]*Department of Chemistry & Biochemistry, University of Arkansas, Fayetteville*
[2]*Maine Medical Centre Research Institue, Scarborough, USA*

1. Introduction

Fibroblast Growth Factor (FGF) signaling plays a vital role in a wide range of cellular responses (Detillieux et al., 2003; Freeman et al., 2003). The activation of FGF Receptor (FGFR) is a crucial step in the diverse FGF signaling pathway (Ayari & Soussi-Yanicostas, 2007). Various intra- and extracellular modulators are involved in the FGF signaling pathway. Anosmin-1, an extracellular matrix associated glycosylated protein, is a newly identified modulator of the FGF-mediated signaling process (Bribian et al., 2006; Gonzalez-Martinez et al., 2004). Anosmin-1 is the product of KAL1 gene. The Kallman syndrome (KS) is a manifestation of the different loss-of-function mutations in the KAL1 gene. The most characteristic features of the Kallman syndrome are anosmia (lack of smell) and hypogonadotrophic hypogonadism (Maestre, 1856; Kallmann et al., 1944). The biological role(s) of Anosmin-1 has been studied both at the cellular and at the biochemical level. Anosmin-1 is suggested to be involved in cell adhesion, cell migration, cell proliferation as well as cell differentiation (Andrenacci et al., 2004; Andrenacci et al., 2006; Ardouin et al., 2000; Bribian et al., 2006; Cariboni et al., 2004; Ernest et al., 2007; Hardelin et al., 1999; Hu et al., 2004; Okubo et al., 2006; Robertson et al., 2001; Rugrali et al., 1996; Soussi-Yanicostas, 1996; Soussi-Yanicostas et al., 1998; Soussi-Yanicostas et al., 2002; Yanicostas et al., 2008;). Anosmin-1 is shown to be a heparin binding protein and formation of the heparin-Anosmin binary complex is believed to be crucial for the function of the protein (Bulow et al., 2002). Interestingly, recent reports suggest a direct interaction between the N- terminus of Anosmin-1 and the FGF-2/FGFR-1/HS ternary complex (Hu et al., 2009; Hu & Bouloux, 2010).

The structure of Anosmin-1(Figure 1) comprises of an N-terminal cysteine-rich domain (CR), whey acidic like-protein domain (a-WAP), four fibronectin type III (FnIII) repeats and C-terminal histidine rich region. Recent reports suggest direct interactions of some of the structural domains of Anosmin-1, including a-WAP, with the fibroblast growth factor receptor(s). Although the Anosmin-FGFR binding interface is still not mapped, it is strongly believed that the interactions of Anosmin-1 with FGFR(s) regulates the activation of the receptor.

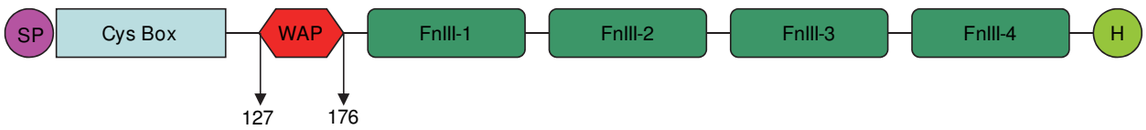


Fig. 1. Schematic representation of human full length Anosmin-1 protein with all the domains, the a-WAP domain labeled in red color spans from 127 to 176 amino acid.

Evolutionarily, Anosmin-1 sequences show the highest conservation within the N-terminal a-WAP and the FnIII-1 domain. Therefore, it is contemplated that these structural domains are functionally significant. The WAP domain of Anosmin-1 (a-WAP) contains 8 cysteine residues which are disulphide bonded to form the four-disulphide core (FDSC) (Figure 2). Most of the proteins containing WAP domain exhibit protease inhibitory activity and consequently regulate the cell proliferation, cell differentiation and tissue remodeling processes (Whitlock et al., 2005). In marked contrast, Anosmin-1 containing the a-WAP domain has been shown to promote the uPA (uro-plasminogen activator) proteolytic activity (Hu et al., 2004).

A.

MVPGVPGAVLTLCLWLAASSGCLAAGPGAAAARRLDESLSAGSVQRARCASRCLSLQITRISAFFQHFQNNGSL
VWCQNHKQCSKCLEPCKESGDLRKHQCQSFCEPLFPKKSYECLTSCEFLKYILLVKQGDCPAPEKASGFAAACV
ESCEVDNECSGVKKCCSNGCGHTCQVPKTLYKGVPLKPRKELRFTELQSGQLEVKWSSKFNISIEPVIYVVQRR
WNYGIHPSEDDATHWQTVAQTTDERVQLTDIRPSRWYQFRVAAVNVHGTRGFTAPSKHFRSSKDPSAPPAPANL
RLANSTVNSDGSVTVTIVWDLPEEPDIPVHHYKVFWSWMVSSKSLVPTKKRRKTTDGFQNSVILEKLQPDCDY
VVELQAITYWGQTRLKSAKVSLHFTSTHATNNKEQLVKTRKGGIQTQLPFQRRRPTRPLEVGAPFYQDGQLQVK
VYWKKTEDPTVNRYHVRWFPEACAHNRTTGSEASSGMTHENYIILQDLSFSCKYKVTVQPIRPKSHSKAEAVFF
TTPPCSALKGKSHKPVGCLGEAGHVLSKVLAKPENLSASFIVQDVNITGHFSWKMAKANLYQPMTGFQVTWAEV
TTESRQNSLPNSIISQSQILPSDHYVLTVPNLRPSTLYRLEVQVLTPGGEGPATIKTFRTPELPPSSAHRSHLK
HRHPHHYKPSPERY

B.

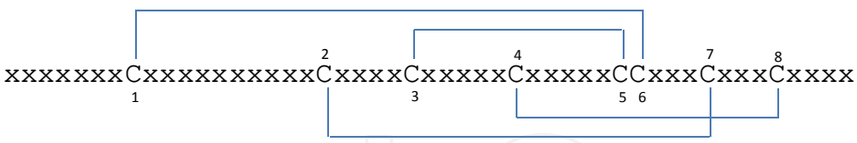


Fig. 2. Panel A. depicts the amino acid sequence of full length Anosmin-1. Residues, 127-176, (shown in red) spans the a-WAP domain with eight conserved cysteines involved in the intra FDSC(four-disulphide core) motif that are highlighted in blue.
Panel **B**. shows the highly conserved intramolecular disulphide bonding pattern in the a-WAP domain.

Some of the mutations in the a-WAP domain are believed to lead to the loss of functions of Anosmin-1. Missense mutations of C172R or C163Y in the KS patients are predicted to result in the disruption of the conserved disulphide bonds and consequently affecting the stability and folding of the protein (Hu et al., 2004). The present study is aimed at understanding the structure-function relationship of the a-WAP domain of Anosmin-1. Therefore, significant quantities of protein(s) are required to embark on detailed structure-function relationship studies. With the advent of the recombinant DNA technology, over-expression of recombinant proteins in heterologous hosts has been rendered easy. Among the expression hosts, *Escherichia coli* (*E.coli*) is an apt choice because it is one of the best

studied model systems which is not only easy to handle but is also known to produce recombinant proteins in high yields.

In general, proteins of interest are cloned into a suitable expression vector and overexpressed under selected induction conditions. One of the most successful bacterial expression vectors available is the pGEX system. We have quite successfully cloned a number of genes/DNA segments into this vector and expressed them as soluble GST fusion proteins in milligram quantities from a liter of bacterial culture. In this chapter, we summarize the procedure used to clone, overexpress and purify the a-WAP domain of Anosmin-1. The method described can be applied to most other recombinant proteins that are difficult to be expressed in the soluble fraction.

2. Materials and methods

2.1 Cloning

- pGEX-KG' vector (Genentech, USA).
- BL21 (DE3) (New England Bio labs)
- DH5α (New England Bio labs)
- Mini prep plasmid isolation kit (QIAGEN, USA)
- Phusion PCR Master mix Kit (New England Bio Labs)
- Agarose gel electrophoresis buffer: 0.5X TAE buffer: Tris-acetate (20 mM; pH 8.0), EDTA (0.5 mM).

2.2 Protein expression

- LB (Luria-Bertani) medium (EMD Chemicals Inc.) for bacterial cultures: Dissolve 25g in 1L deionized water and autoclave at standard conditions.
- Ampicillin (Cellgro®): stock solution of 100 mg/ml was prepared and stored at -20 °C.
- Isopropyl β-D-thiogalactoside (IPTG) (Research Products International Corp.): 1M stock solution was prepared and stored at -20 °C.
- SDS PAGE electrophoresis buffer: 1X Tris-glycine buffer – trizma base (25 mM), glycine (50 mM), SDS (0.1%), final pH pH 8.3.
- Staining solution for SDS PAGE: Coomassie Brilliant Blue R-250 (0.25%) in destaining solution.
- Destaining solution for SDS PAGE: methanol (30%), glacial acetic acid (10%).
- 4X SDS gel-loading Buffer: Tris.HCl (100 mM; pH 6.8), SDS (4%), bromophenol blue (0.2%), glycerol (20%), β-Mercapthoethanol (200 mM).

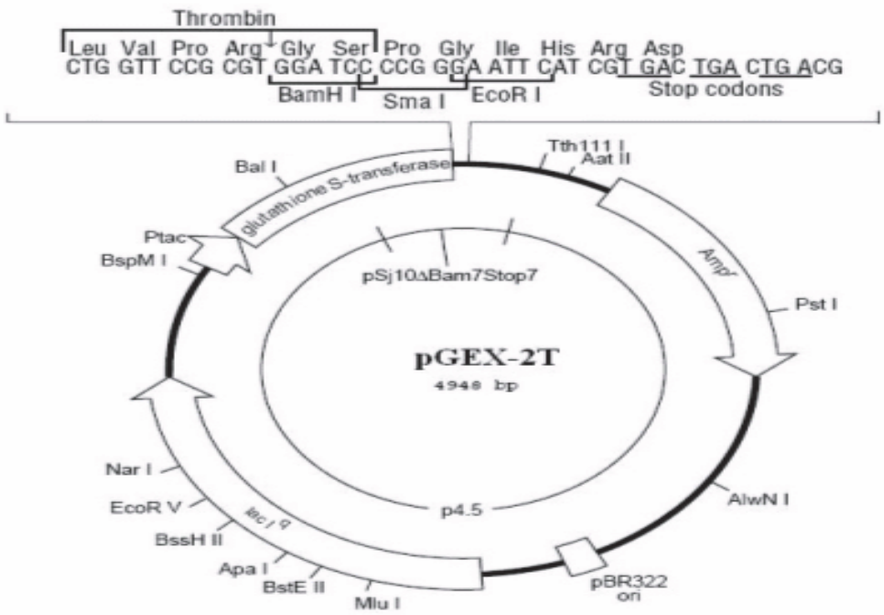
2.3 Protein purification

- Phosphate Buffered Saline (1xPBS): Na_2HPO_4 (10 mM), KH_2PO_4 (2 mM), NaCl (137 mM), KCl (2.7 mM), final pH 7.2.
- GSH Sepharose® beads (GE Health care) are used as affinity based chromatography.
- Thrombin (Sigma) is dissolved in 2mL of 1xPBS pH 7.2 to obtain a concentration of 1U/μL.
- Thermo scientific FH 100 Peristaltic Pump was used for loading the buffers on the column.
- Purification was monitored using UV detector from BIORAD.

2.4 Cloning of a-WAP into the pGEX-KG expression vector

The pGEX-KG (Figure 3) vector from GE Healthcare was used for the expression of the gene of interest as the fusion protein with an N-terminal sequence of glutathione S-transferase (GST). The fusion protein can be cleaved with thrombin (cleavage sequence Leu-Val-Pro-Arg-↓-Gly-Ser) to obtain the recombinant protein with a 15 extra amino acids at the N-terminus.

The multiple cloning site (MCS) of this pGEX-2T contains only three sites for cloning: BamH1, SmaI, and EcoRI. To enhance the MCS, the pGEX-2T vector was linearized with EcoRI and was ligated to a cassette containing an expanded multiple cloning site (MCS) to produce a vector termed pGEX-KG'. The sequence of the MCS is shown (Figure 4). Therefore, the sequence beyond the cleavage site for thrombin in the pGEX-KG' vector will code for: Leu-Val-Pro-Arg-↓-Gly-Ser-Pro-Gly-Ile-Ser-Gly-Gly-Gly-Gly-Gly-Ile-Asp-*Ser-Met*-Gly-Arg-*Leu-Glu*-Leu-Lys- (continues further along). The NcoI site cuts between the Ser-Met residues and the XhoI site cuts between the Leu-Glu residues. The NcoI and XhoI sites were used for cloning the a-WAP gene of Anosmin-1.



pGEX-2T vector carries ampicillin resistance, pBR322 origin of replication, Lac promoter and GST tag at the 5' end of the MCS.

Fig. 3. Vector Map of pGEX-2T

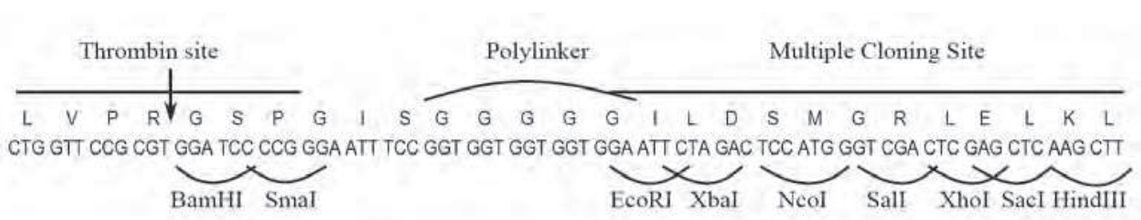


Fig. 4. MCS of the pGEX-KG vector

2.5 PCR-based cloning of a-WAP

The gene of interest was amplified from the optimized *E.coli* codon human full-length Anosmin-1 stabilized in pUC19 (GeneArt, Life technologies, USA) by using gene-specific primers a-WAP-FP (5'ACT GCCATGGTGCTGGTGAAACAGG3') and a-WAP-RP (5' ACTGCTCGAGTTTCGGAACCTGACAG 3'). The PCR conditions were as described in the manufacturer's protocol with the following modifications: DNA (50 ng -200 ng), forward and reverse primers (0.5μM each), 2xPhusion Flash PCR Master Mix (1x) (New England Biolabs) were added to a 20 μl reaction mixture. The samples were incubated in a Master cycler Gradient (Eppendorf) for an initial denaturation (10sec.; 98°C) followed by 30 cycles each including denaturation (2sec.; 98°C), annealing (5 sec.; 62°C), extension (15sec.; 72°C), and the final extension (1min.; 72°C). The PCR product was checked by agarose gel electrophoresis.

The PCR product was purified using a Qiagen PCR purification column according to the manufacturer's instructions to get rid of the unused primers, nucleotides and other material. The purified PCR product and pGEX-KG vector were subjected to double digestion with NcoI and XhoI. The digested PCR product and the vector were gel purified and were subjected to ligation reaction using the Rapid Ligation kit from MBI Fermentas. Conditions for agarose gel resolution, recovery of insert from gel slices, quantification, and ligation were as per standard cloning protocols. Ligation mixtures were used for transformation of DH5α chemical competent cells (New England Biolabs). Recombinant plasmids were purified from bacterial colonies and subjected to restriction analyses and DNA sequencing to confirm their identity.

2.6 Expression and purification of recombinant GST-a-WAP fusion protein in *E.coli*

The plasmid containing recombinant a-WAP was transformed into *E. coli* BL21 (DE3) cells. A single colony was inoculated into 10 ml LB broth containing ampicillin (100μg/ml) and incubated at 37 °C overnight. For large scale expression, LB broth containing ampicilin (100μg/ml) was inoculated with 5% (v/v) overnight culture and incubated at 37 °C and 250 rpm. Once the OD_{600} reached 0.8, 1 mM IPTG was added to the cells, which were further incubated for four hours. Cells were harvested at 6000 rpm for 20 minutes at 4 °C using Beckman JA-10 rotor. The pellets were washed using 1x PBS and either were used immediately or stored at -20 °C.

E. coli cells containing recombinant GST-a-WAP protein were resuspended in 20 mL of 1xPBS. The suspension was lysed using French press at a pressure of 1000 psi. Cell debris was removed by centrifugation at 20000 rpm for 20 min. at 4°C using Beckman JA-20 rotor.

The supernatant was loaded on to the pre-equilibrated (1xPBS) GSH-Sepharose column at a flow rate of 1ml/min. After loading the supernatant on the column, the column was washed with 1xPBS until a flat baseline was reached. Quantity of thrombin to be used for complete cleavage was standardized based on experiments involving in-solution cleavage of GST-a-WAP. For all further purification of a-WAP, thrombin was added at the ratio of 1U/250μg of recombinant GST-a-WAP protein onto the column for an on-column cleavage, which was incubated on the rocker (VWR) at slow speed (45-60 rpm) at room temperature for 24hrs. a-WAP was eluted using 1xPBS and concentrated using Amicon or Millipore concentrators.

2.7 Determination of protein concentration

The concentration of the protein was estimated by measuring absorbance at 280nm (ε_{280nm} = 1.303) using Agilent spectrophotometer.

2.8 MALDI-MS analysis of the a-WAP

Prior to MALDI-TOF (Bruker Daltonics) analysis, recombinant a-WAP (~50-100 µg) sample was desalted by passing through "ZIP™" tips (C-18 matrix). The theoretical molecular weight of a-WAP was calculated using ProtParam tool from Expasy and was found to be 7317.3 Da which was compared with the experimental value obtained from MS-Analysis.

2.9 Circular Dichorism (CD) analysis

CD data were recorded as an average of fifteen accumulations at room temperature using a Jasco J-720 spectropolarimeter. Far UV CD spectrum of a-WAP (166 µM) in 1xPBS pH 7.2 was recorded using a quartz cell of 0.1 mm pathlength in the standard sensitivity mode with a scan speed of 50 nm per minute. Appropriate blank corrections were made in the CD spectrum. The CD data are expressed as molar ellipticity ($deg.cm^2.dmol^{-1}$).

2.10 Differential scanning calorimetry

Heat capacity of a-WAP was measured as a function of temperature at pH 7.2 using NANO DSCIII with a ramping temperature of 1^0C/min from 10^0C to 90^0C. Thermal denaturation scans were performed using a protein (a-WAP) concentration of ~ 160 µM. The protein solution was degassed in 1xPBS, pH 7.2 prior to acquisition of DSC data. Both the heating and cooling cycles were recorded to examine the reversibility of the thermal unfolding process.

3. Results

3.1 PCR amplification and cloning of a-WAP

Human full length Anosmin-1 gene nucleotide sequence was codon optimized with respect to *E.coli*. The Anosmin-1 gene was cloned in the pET-20b vector. The expression yield(s) of the protein was not as expected and therefore each individual structural domain of the Anosmin-1 gene was cloned and overexpressed in *E.coli*. The significantly different structural and also functional properties of the a-WAP domain of Anosmin-1, as compared to the WAP domains in other proteins, directed our interest to embark on the cloning, overexpression, purification, and biophysical characterization of the a-WAP domain. The actual size of the a-WAP domain is 150 bp but after the PCR amplification the product size increased to 170 bps due to the addition of 10bps at the 5′ and 3′end that includes the desired restriction sites. This 170 bp NcoI – XhoI fragment was cloned into pGEX-KG′ expression vector (please see methods for a description of this expression vector) so that the coding sequence of a-WAP would be in-frame with the GST coding sequence of this vector. This would allow the expression of a GST-a-WAP fusion protein from the pTac promoter (T7 and Lac promoter) of the vector. The colonies obtained after transformation were checked for the positive clones using colony PCR, and plasmid isolated from the positive clones were confirmed by DNA sequencing.

3.2 Overexpression of a-WAP

E. coli BL-21 (DE3) cells transformed with pGEX-KG′-a-WAP were grown in the presence of 1mM IPTG to induce the overexpression of the GST-a-WAP fusion protein. A protein of ~32

kDa appeared to be induced in cultures grown in the presence of IPTG (Figure 5). The cells obtained after induction were collected to check for the presence of a-WAP in either the soluble fraction or in the pellet as inclusion bodies. Therefore, the bacterial pellet obtained from induced cells was subjected to cell lysis and both the supernatant and sonicated pellet fractions collected after centrifugation was resolved under reduced denaturation conditions, showed that the GST-a-WAP was totally expressed in the soluble fraction (Figure 5).

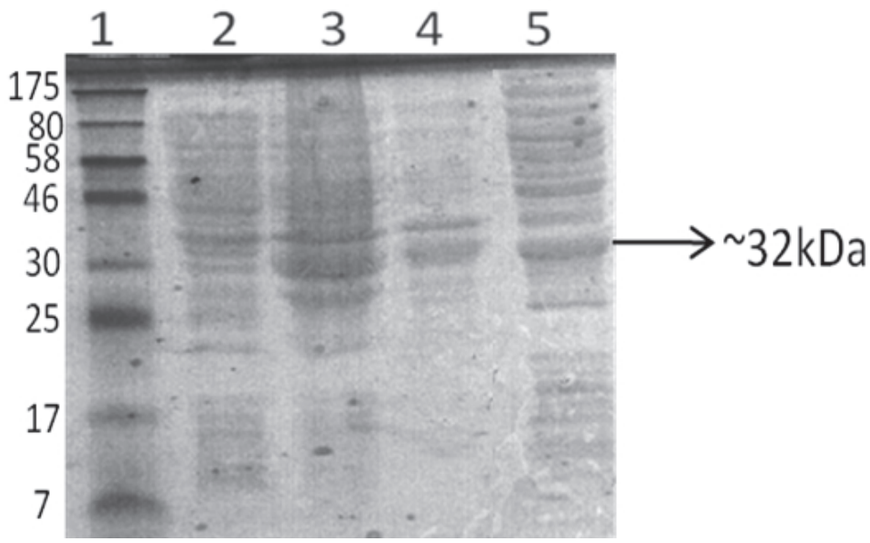


Fig. 5. Induction and purification of recombinant a-WAP. Cell lysates obtained before and after induction with IPTG were resolved on 15% SDS PAGE and was stained with Coomassie Brilliant Blue – R 250. Lane 1: Broad range molecular marker; Lane 2: lysate of uninduced culture; Lane 3: lysate of induced culture showing a overexpressed band ~32kDa; Lane 4: Pellet of the induced cells after the sonication followed by centrifugation; Lane 5: Supernatant obtained from induced cells after the sonication followed by centrifugation containing GST-a-WAP.

3.3 One step purification of a-WAP

a-WAP was purified to homogeneity by a single-step procedure using glutathione-sepharose affinity chromatography (Figure 6). The expression yield of GST-a-WAP was ~16mg/L of the induced *E.coli* culture. The GST-a-WAP fusion protein recovered from the affinity column was subjected to in-solution thrombin cleavage. It was observed that 1U of thrombin cleaved 250 µg of GST-a-WAP. The yield of recombinant a-WAP after thrombin cleavage was ~4mg/L of the induced *E.coli* culture(s). The purified a-WAP migrated as a single band corresponding to a molecular mass of ~7 kDa on SDS-PAGE under reducing conditions.

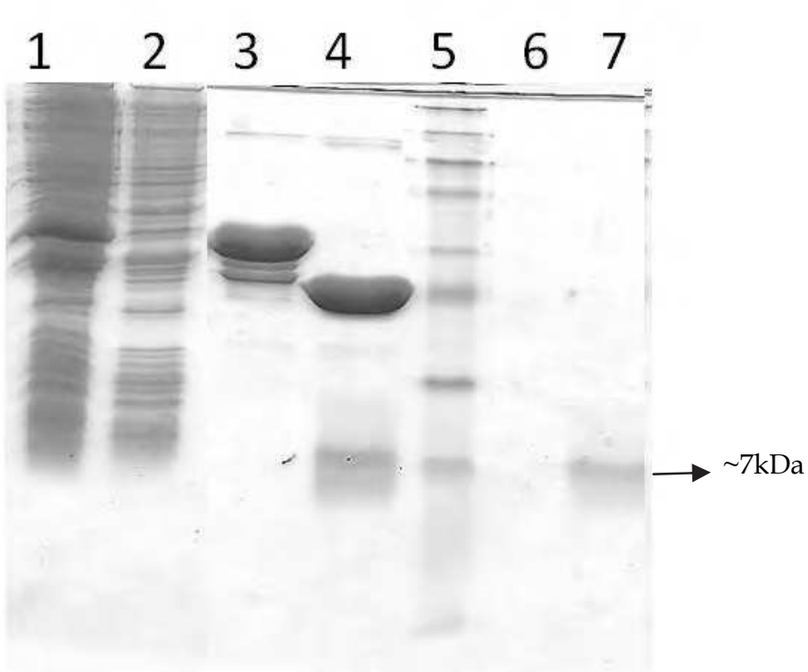


Fig. 6. Purification profile of a-WAP: Samples after each step of purification were resolved on 15% SDS-PAGE stained with Coomassie Brilliant Blue – R 250 Lane 1: Supernatant obtained after sonication; Lane 2: Flow through obtained after binding of the supernatant with 1xPBS pH 7.2; Lane 3: Sample collected upon elution with 10mM GSH(reduced); Lane 4: Sample of in-solution thrombin cleaved GST-a-WAP containing bands corresponding to 26kDA GST and also ~7kDA of a-WAP; Lane 5: Molecular weight marker ; Lane 6 : Empty lane ; Lane 7: Sample obtained by eluting the on-column thrombin cleaved (Incubated at room temperature for 24hrs) a-WAP using 1x PBS at pH 7.2.

3.4 MALDI-MS analysis of a-WAP

This is one of the most reliable and accurate method for the determination of molecular mass of a protein based on the ion mass-to-charge ratio. The protein obtained after purification was in 1X PBS buffer pH 7.2. The protein sample needs to be desalted prior to mass analysis because inorganic salts are known to significantly interfere with the ionization process in the mass spectrometry experiment(s). The monoisotopic mass of purified a-WAP from MALDI-MS analysis was observed to be 7312 Da (Figure 7). Theoretical molecular weight of a-WAP was calculated from the PROTPARAM tool from EXPASY was found to be 7317.3Da.

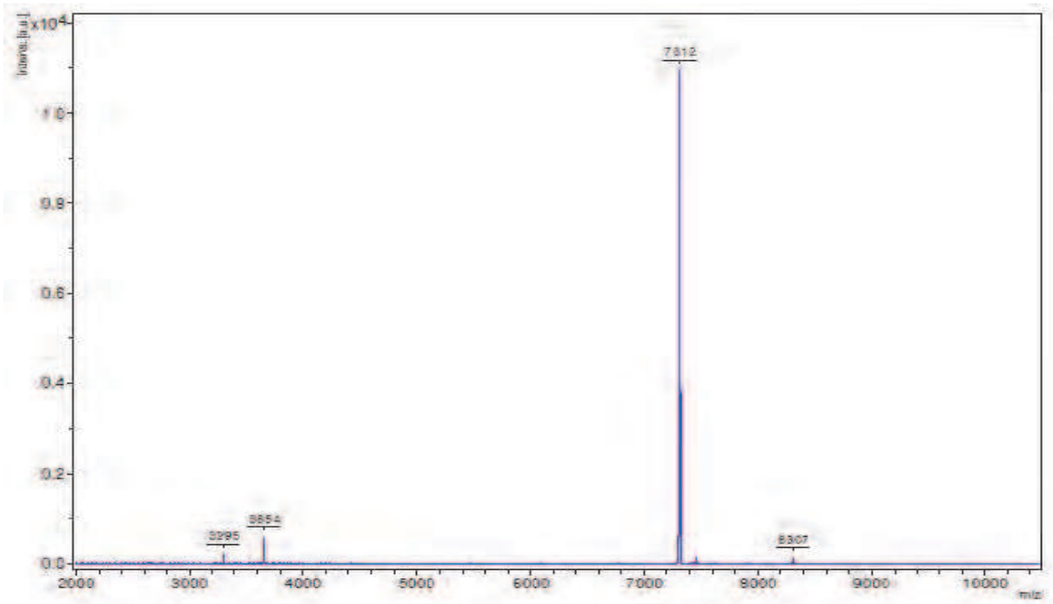


Fig. 7. MALDI-MS analysis of the a-WAP domain. a-WAP MALDI-MS analysis of the desalted a-WAP domain showed a peak that corresponds to a monoisotopic mass of 7312 Da.

3.5 CD analysis of a-WAP

Far UV Circular Dichorism spectroscopy is a powerful technique that is used for assessing the secondary structural elements in proteins. Far UV CD spectrum of the a-WAP showed the characteristic double minima centered at 208 nm and 222 nm suggesting that the backbone of the protein is predominantly in a helix conformation. Interestingly, the aromatic amino acid contributions to the far UV CD spectrum, in the wavelength region of 225 nm to 230 nm, are conspicuously missing. This observation corroborates well with the absence of tryptophan and tyrosine residues in the a-WAP domain of Anosmin-1(Figure 8).

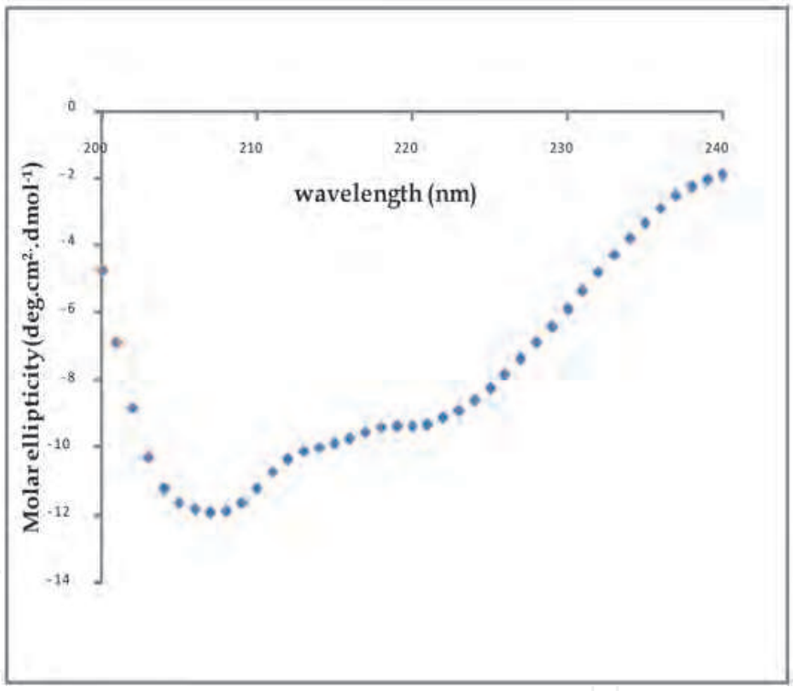


Fig. 8. CD Spectral Analysis of a-WAP. Far-UV CD profile of 166 μM a-WAP was recorded in 1xPBS at pH 7.2 at 25^0C showed that the majority of the protein shows helicity.

3.6 DSC Analysis of a-WAP

Folding and unfolding of proteins are accompanied by heat effects which are largely exothermic in nature. Heat of unfolding measures the enthalpy of the process. Direct measurement of heat of unfolding was examined by differential scanning calorimetry. The change in enthalpy as function of temperature yields the specific heat associated with protein unfolding. It can be observed that the T_m (the temperature at which 50% of the protein population exists in the denatured state(s) is ~ 75.8 C. Interestingly, the thermogram representing the unfolding is broad suggesting that the two or more states of the protein are in equilibrium with each other (Figure 9). Such thermal transitions are not uncommon for proteins, such as the a-WAP domain of Anosmin-1, which are rich in disulfide bonds.

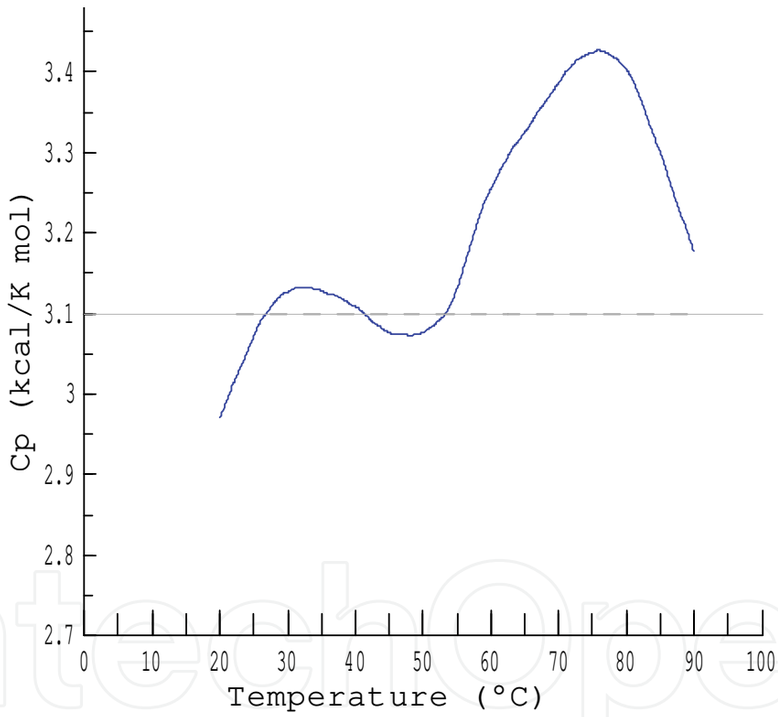


Fig. 9. DSC profile of a-WAP. DSC profile obtained for 166 μM a-WAP in 1x PBS pH 7.2. The T_m for the transition from the folded to the denatured state(s) of the a-WAP is 75.8^{0}C. The change in enthalpy and entropy for the denaturation of the a-WAP are 7.5292kcal/mol and 0.0216kcal/(K mol), respectively.

4. Discussion

Recently, there is an increasing interest in understanding the structure and function of proteins. This endeavor warrants the design of avenues that facilitate cost effective production of recombinant proteins. To-date, *E. coli* has been the most suitable and commonly used heterologous host for production of recombinant proteins. However, its use requires two steps: introduction of DNA in to the host cell and efficient expression of the target protein. Therefore, an appropriate choice of the vector needs to be made to overexpress the recombinant protein(s) of interest. To-date, Anosmin-1 has been expressed only in eukaryotic hosts. To reach an abundant and inexpensive expression of Anosmin-1, for the first time an attempt was made to express the protein in *E.coli*. As the expression of the full length gene did not result in satisfactory yields of the protein in soluble fraction, the cloning and expression of independent structural domains of the protein was attempted in a quest to obtain viable yields. Of all the structural domains of Anosmin-1, the N-terminal a-WAP domain has been suggested to play an important role in the function of the protein. In this context, in the present study, the a-WAP domain was cloned and overexpressed in *E.coli*. All the WAP family proteins exhibit proteolytic activity but the a-WAP domain of Anosmin-1 shows anti-protease activity (Hu et al., 2004). Interestingly, mutations of the conserved cysteine residues in the a-WAP domain result in the Kallman syndrome. Therefore, detailed characterization of the a-WAP domain of Anosmin-1 is crucial for understanding the molecular basis underlying the Kallman syndrome.

To-date, a-WAP domain from Anosmin-1 has been expressed in S2 cells of Drosophila with a yield only sufficient for detection by Western blots. The very meager expression yields obtained therein precludes complete structural characterization of the protein using biophysical techniques such as X-ray crystallography or multidimensional NMR techniques which require substantially high protein concentrations. In this context, the high yields (~ 4 mg- 5mg/L of the bacterial culture) of the a-WAP domain obtained using our cloning and overexpression strategy is significant. Most importantly, the recombinant a-WAP domain, overexpressed and purified using our method, is well-folded with the backbone of the protein predominantly in the helical conformation.

In general, overexpression and purification of small cysteine-rich proteins in heterologous bacterial hosts such as *E.coli* is considered very challenging. However, the results of this study clearly show that optimization of the codons and proper choice of the protein affinity tag are crucial for the high expression yields of small and cysteine-rich proteins such as the a-WAP-domain of Anosmin-1. In our experience, the GST-tag considerably increases the expression yield and most importantly helps in the circumvention of problems generally associated due to lack of elaborate post-translational machinery in *E.coli*. In our opinion, the GST tag has a 'chaperoning' effect on the folding of the cysteine-rich a-WAP domain. This aspect is quite significant because most of the mammalian proteins are known to be produced as inclusion bodies when expressed in *E.coli*. In addition, from a structural biology point-of-view, production of GST-fused small molecular weight proteins is advantageous because the GST-tag does not significantly interfere with the different steps involved in the determination of 3D solution structures of proteins using multidimensional NMR experiments. We believe that the cloning and expression strategy employed in this study will be generally applicable to other small molecular weight, cysteine-rich proteins.

5. Conclusions and future trends

Anosmin-1 is known to play a crucial role in the regulation of the FGF signaling process. However, the exact mechanism by which Anosmin-1 regulates the FGF-induced signaling is still not understood. This lacuna in knowledge is primarily due to non-availability of an overexpression system to produce Anosmin-1 or any of its structural domains in high yields. The strategy for cloning and overexpression of the a-WAP domain of Anosmin-1, discussed in this study, is expected to provide the necessary impetus for future structural studies aimed at understanding the role of the a-WAP domain of Anosmin-1 in the regulation of the FGF receptor activation. This is a significant accomplishment because the a-WAP domain of Anosmin-1 is believed to modulate the FGF-receptor interaction due to its high affinity to bind to heparin. Understanding the structure-function of the a-WAP domain of Anosmin-1 is expected to provide important clues for the rational design of therapeutic principles against a multitude of FGF-mediated pathogenesis. In general, the cloning and overexpression strategy reported in this study is expected to be extremely useful for many other small mammalian proteins.

6. Acknowledgement

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7. References

Andrenacci, D., Grimaldi, M. R., Panetta, V., Riano, E., Rugarli, E. I., and Graziani, F. (2006) *BMC Genet* 7, 47

Andrenacci, D., Le Bras, S., Rosaria Grimaldi, M., Rugarli, E., and Graziani, F. (2004) *Gene Expr Patterns* 5, 67-73

Ardouin, O., Legouis, R., Fasano, L., David-Watine, B., Korn, H., Hardelin, J., and Petit, C. (2000) *Mech Dev* 90, 89-94

Ayari, B., and Soussi-Yanicostas, N. (2007) *Dev.Genes.Evol*. 217, 169-175

Bribian, A., Barallobre, M. J., Soussi-Yanicostas, N., and de Castro, F. (2006) *Mol Cell Neurosci* 33, 2-14

Bulow, H. E., Berry, K. L., Topper, L. H., Peles, E., and Hobert, O. (2002) *PNAS* 99, 6346-6351

Cariboni, A., Pimpinelli, F., Colamarino, S., Zaninetti, R., Piccolella, M., Rumio, C., Piva, F., Rugarli, E. I., and Maggi, R. (2004) *Hum. Mol. Genet.* 13, 2781-2791

Detillieux, K.A., Sheikh, F., Kardami, E., and Cattini, P.A. (2003) *Cardiovasc. Res.* 57, 8-19

Ernest, S., Guadagnini, S., Prevost, M. C., and Soussi-Yanicostas, N. (2007) *Gene Expr Patterns* 7, 274-281

Freeman, K.W., Gangula, R. D., Welm, B. E., Ozen, M., Foster. B. A., Rosen, J. M., Ittmann, M., Greenberg, N. M., and Spencer, D. M. (2003) *Cancer Res*. 63, 6237-6243

Gonzalez-Martinez, D., Kim, S.-H., Hu, Y., Guimond, S., Schofield, J., Winyard, P., Vannelli, G. B., Turnbull, J., and Bouloux, P.-M. (2004) *J. Neurosci.* 24, 10384-10392

Hardelin, J. P., Julliard, A. K., Moniot, B., Soussi-Yanicostas, N., Verney, C., Schwanzel-Fukuda, M., Ayer-Le Lievre, C., and Petit, C. (1999) *Dev Dyn* 215, 26-44

Hu, Y., Gonzalez-Martinez, D., Kim, S. H., and Bouloux, P. M. (2004) *Biochem J* 384, 495-505

Hu, Y., Guimond, SE., Travers, P., Cadman, S., Hohenester, E., Turnbull, JE., Kim, SH., Bouloux, PM. (2009) *J.Biol.Chem* 284 (43), 29905-20

Hu, Y., Bouloux, PM. (2010) *Trends in Endocrinology and Metabolism* 21 (6), 385-93

Kallmann, F. J., Schoenfeld, W. A., and Barrera, S. E. (1944) *Am J Ment Defic* XlVIII, 203-236

Maestre De San Jaun, A. (1856) *El Siglo Medico, Madrid* 3, 211

Murcia-Belmonte, V., Esteben, PF., Garcia-Gonzalez, D., De Castro, F. (2010). *J. Neurochem* 115 (5), 1256-65

Okubo, K., Sakai, F., Lau, E. L., Yoshizaki, G., Takeuchi, Y., Naruse, K., Aida, K., and Nagahama, Y. (2006) *Endocrinology* 147, 1076-1084

Robertson, A., MacColl, G. S., Nash, J. A., Boehm, M. K., Perkins, S. J., and Bouloux, P. M. (2001) *Biochem J* 357, 647-659

Rugarli, E. I., Ghezzi, C., Valsecchi, V., and Ballabio, A. (1996) *Hum Mol Genet* 5, 1109-1115

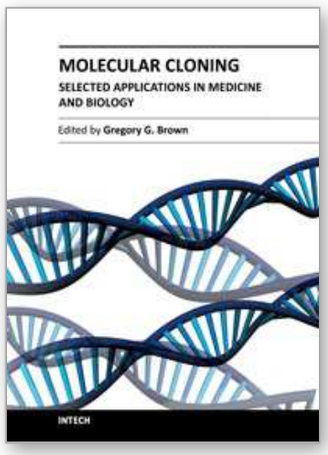
Soussi-Yanicostas, N., de Castro, F., Julliard, A. K., Perfettini, I., Chedotal, A., and Petit, C. (2002) *Cell* 109, 217-228

Soussi-Yanicostas, N., Faivre-Sarrailh, C., Hardelin, J. P., Levilliers, J., Rougon, G., and Petit, C. (1998) *J. Cell Sci.* 111, 2953-2965

Soussi-Yanicostas, N., Hardelin, J. P., Arroyo-Jimenez, M. M., Ardouin, O., Legouis, R., Levilliers, J., Traincard, F., Betton, J. M., Cabanie, L., and Petit, C. (1996) *J. Cell Sci.* 109, 1749-1757

Whitlock, K. E., Smith, K. M., Kim, H., and Harden, M. V. (2005) *Development* 132, 5491-5502

Yanicostas, C., Ernest, S., Dayraud, C., Petit, C., and Soussi-Yanicostas, N. (2008) *Dev Biol* 320, 469-479



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The development of molecular cloning technology in the early 1970s created a revolution in the biological and biomedical sciences that extends to this day. The contributions in this book provide the reader with a perspective on how pervasive the applications of molecular cloning have become. The contributions are organized in sections based on application, and range from cancer biology and immunology to plant and evolutionary biology. The chapters also cover a wide range of technical approaches, such as positional cloning and cutting edge tools for recombinant protein expression. This book should appeal to many researchers, who should find its information useful for advancing their fields.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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