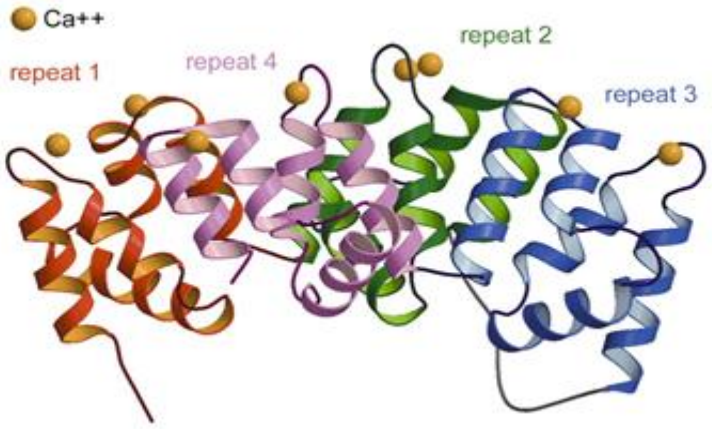

Annexin 2 & P11-Expression, Purification and Biophysical Characterization.

Under guidance of :-

Guide-
Prof. Avadhesha Surolia, Honorary Professor.
Molecular Biophysics Unit, Indian Institute of Science, Bangalore.

Co- Guide-
Prof. Vikas Handa, Associate Professor
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Submitted by
Name - Ms. Kritika Kaur
Roll No. - 301301007
Batch 2013-2015
M.Sc. Biotechnology (Medical)

CERTIFICATE

I hereby certify that the work which is being presented in the M.Sc. Biotechnology (Medical) Project Report entitled *'Annexin 2 and P11 Expression, Purification and Biophysical Characterization'*, by Ms. Kritika Kaur, in partial fulfillment of the requirements for the award of the degree to the M.Sc. Biotechnology (Medical) is a record of her original bonafide work carried out during a period from January 2015 to June 2015. She has carried out the reported work under my guidance and supervision at Indian Institute of Science, Bangalore, and that it has reached the requisite standards for submission as dissertation report.

Moreover, to the best of my knowledge and belief, the results presented in this Project Report and the associated content has not been submitted by me for the award of any other degree elsewhere.

Dr. Avadhesha Surolia
Honorary Professor, Molecular Biophysics Unit
Indian Institute Of Science, Bangalore

Dr. Vikas Handa
Assistant Professor,
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Dr. S. S. Bhatia
Dean of Academics Affairs
Thapar University, Patiala.

CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the report entitled ***"Annexin 2 and P11- Expression, Purification and Biophysical Characterization"*** in partial fulfillment of the requirement for the award of degree of Masters of Science in Biotechnology (Medical) to Thapar University, Patiala is my own work during the period of six months from Ianuary 2015 to June 2015, under the supervision of Dr. Avadhesha Surolia, Honorary Professor, Molecular Biophysics Unit, Indian Institute of Science, Bangalore and Dr.Vikas Handa, Assistant Professor, Department of Biotechnology, Thapar University, Patiala.

I have not submitted the matter embodied in this report for any other degree.

Kritika.

Date :- 15th July 2015

Name - Kritika Kaur

Roll No.- 301301007

It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

Date :-

Avadhesha Surolia

Dr. Avadhesha Surolia
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Kritika.

Place :- MBU, Indian Institute of Science, Bangalore.

Date:- 15th July 2015

Name of the canditate

Ms. Kritika Kaur

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Abstract :-

Annexin 2 fits in with the class of proteins that tie to acidic phospholipids in a calcium subordinate way which has prompted the proposal that it capacities in exocytosis, endocytosis and cell-cell adhesion. This makes this protein an interesting molecule to study in detail. With a specific end goal to portray the interactome of annexin II , we have over expressed, purified & characterized annexin II & one of its communicating partner S100A10 (P11). We have additionally affirmed the association between annexin II & S100A10 (P11) by Co-Immunoprecipitation test.

Introduction

The annexins[(Swairjo MA, Seaton BA,1994] are a group of Ca 2+ binding proteins which bind to acidic phospholipids, and are further identifiable as individuals from this family, by the presence of same amino acid sequence forming a special kind of folds called the'annexin fold [Geisow MJ,1986]. The name annexin [Geisow MJ, *et al.*,1986] which starts from the property of the individuals from this family to add phospholipid, has been recommended as a premise for a typical terminology for these proteins. To date,thirteen annexins have been purified and examined and these proteins have been found in every single mammalian cell but erythrocytes. Annexins have additionally been portrayed in life forms going from well evolved creatures i.e. mammals to moulds and plants. Most annexins are abundantly intracellular proteins and may involve around 0.5-2% of aggregate cell proteins. Ability of Anneixn II to bind to calcium and cell membrane has helped us to understand the protein's role in membrane trafficking events like cell-cell adhesion, exocytosis and endocytosis.

Annexin has been shown to exist as monomer and oligomeric forms like homodimer, heterodimer and heterodimer. It is present majorly in the cytosolic sites of a cell. The monomer is usually a 36 KDa protein and heterodimer is composed of one unit of annexin II with one unit of 3-Phosphoglycerate Kinase and a heterotetramer is made up of two units of annexin II and two units of P11 (S100A10). These different forms are scattered in the cell non uniformly, usually the heterotetrameric form is found outside the cell near cell membrane.The development of the heterodimer results in the relationship of the complex with the nucleus where it has been seen to control DNA polymerase α. In the protein complex, annexin II is considered to be the heavy chain and P11 (S100A10) is considered to be the light chain.

Annnexin II has structure which has four 70 amino acid's conserved domains, these conserved domains are conserved in all forms of annexins found throughout and these 70 amino acid's fold in such a way forming a special kind a fold called "annexin fold", whose crystal structure depicts that each domain has five α helices. This special annexin fold is called as "Endonexin Fold". These annexin folds are repeated four times in almost all annexin's isolated and characterized till date except annexin VI, an exception showing eight folds and molecular weight falling in the category of 60-80KDa unlike other annexin family proteins.

Annexin II binds to other partner P11 (S100A10) forming a pseudo two fold symmetry.

All annexin's bind to phospholipids which curves out to be a fundamental property of annexin protein family with different Kd values. For instance, annexin I binds to phosphatidylserine liposomes with a lower Kd (Ca 2+) than annexin V however the binding affinity of annexin V for phospholipid liposomes is much higher than that of annexin I. Generally, the annexins have been found to bind to phosphatidic acid, phosphatidylserine and phosphatidylinositol liposomes with increasing Kd (Ca 2+) [Blackwood RA, Ernst JD,1990]. Studies with chimeric annexins have proposed that the first annexin repeat may regulate the general activity for phospholipid binding however is presumably not included in deciding the Ca 2+ prerequisite for phospholipid binding.

A few basic components of the annexins are penetrating through a layer entering part for the protein. For example, the protein contains a high density of charged and polar groups where layer penetration is required to happen. Moreover, the annexin helices that are proposed to span the layer are by 6-8 residues shorter than needed for known layer spanning over membrane helices. Moreover, the pseudo 2-fold of axis symmetry, which has been proposed to take part in channel function, is hindered by salt scaffolds/bridges.

Tissue content of annexin II has been reported in avian and mammalian cells. The protein stays absent in heart,smooth muscles, skeletal muscles,liver, platelets, erythrocytes. Low concentratio of protein is reported in brain while intermediate concentrations are reported in adrenal gland, kidney and spleen. Intermediate and high concentrations of annexin II present in different cell types such as epithelial cells of skin, respiratory and intestinal cells, endothelial cells of blood type and chondrocytes of connective and cartilage tissue. Other blood types rich in annexin proteins are fibroblasts, macrophages, splenocytes and HeLa cells.

The relative content of annexin II monomer and annexin II tetramer vary throughout the cells. Thymus contains almost annexin II monomer where as the ration of annexin II tetramer/annexin II monomer varies from 50% in fibroblasts to 100% in intestinal epithelium. It has also been shown that the expression of P11 and annexin II monomer is not always coordinated.

Literature's Review

Annexins are a group of proteins that binds acidic phospholipids in the vicinity of Calcium. They are present on chromosome 15q22.2. The cooperation of these proteins with lipid bilayer has lead us to the conclusion that these proteins take a part in membrane trafficking, for example, exocytosis, endocytosis and cell-cell adhesion[Waisman DM,1990]. Annexin II has demonstrated to exist as a monomer, heterodimer or heterotetramer[Waisman DM,1990]. The capacity of annexin II tetramer to connect secretory granules has served to reason that it is included in exocytosis. It has additionally been found to interact with metastatic cells furthermore connects metastatic tumor cells with normal cells. Most annexins are rich intracellular proteins and may be present around 0.5-2% of aggregate cell proteins [Waisman DM,1990]. Regularly, annexin's atomic weight falls in the scope of 30-40 kDa with a special case of annexin VI which is a 68 kDa protein.. Various different functions of the protein have been reported like cell-cell adhesion, inhibition of phospholipase A, inhibitor of blood coagulators and transducers for signals for differentiation and mitosis.

Annexin II (AII) is an abundant protein which exists as a monomer (AIIm, 36 kDa), a heterodimer (AIId) or a heterotetramer (AIIt)[Waisman DM,1990]. The heterodimer is made up of one unit of AII and one unit of 3-phosphoglycerate kinase and the heterotetramer is made up of two AII units and two 11 kDa units of S100A10 (P11). The AII monomer is essentially cytosolic. Formation of heterotetramer with nucleus has been seen to function as DNA polymerase α [Waisman DM,1990]. . Relationship of a tetrameric complex prompts spanning of plasma layers of two cells. The AII gene is growth regulated. In vitro annexin II's funtions have been studied over and experiments have demonstrated that it requires calcium for all its functions.[Kassam *et al*,1997& Waisman DM,1990].

Annexin II has two main domains- N terminal domain and C terminal domain. N terminal domain of annexin II play a regulatory role in binding of chromaffin granules and it has also been seen that binding of P11 to Annexin II reduces its interactions with chromaffin granules. C terminal of annexin 2 consists of calcium binding sites, Phospholipid binding sites, F-actin and heparin binding sites.

F- actin is a multi globular protein found in eukaryotic organisms which generally helps in cellular mobility and contraction of cells during cell division.

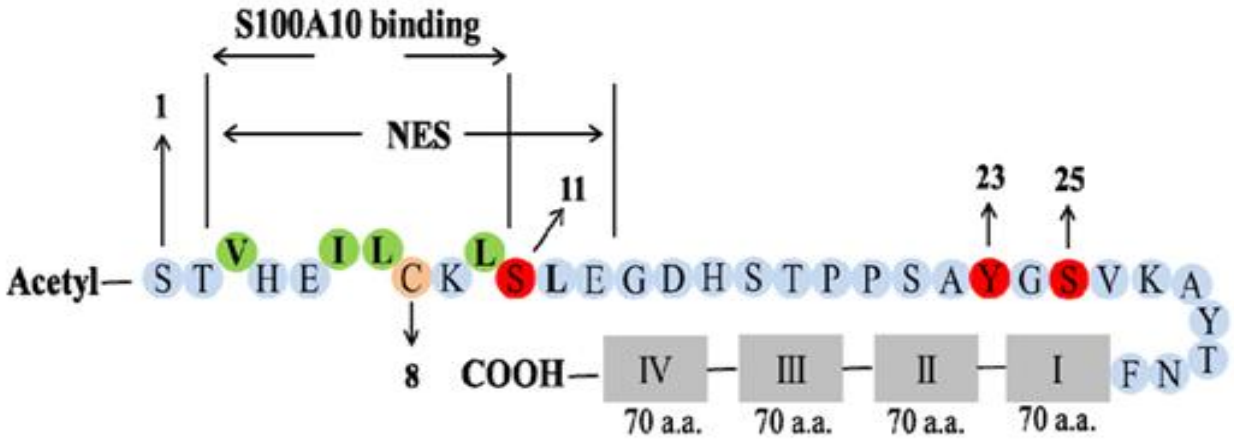

Fig.1- Structure of annexin 2 monomer- The figure shows four 70 amino acids conserved domain and phosphorylation sites. Also sites responsible for calcium binding and phospholipid binding.[Waisman DM,1990]

- Ser 11& Ser 25- PKC phosphorylation site.
- Val 3, Ile 6, Leu 7 and Leu 10- S100A10 Binding hydrophobic residues.
- Cys8- Redox active cysteine.
- Carboxyl domain- calcium, phospholipid, membrane, F-actin and heparin binding site.

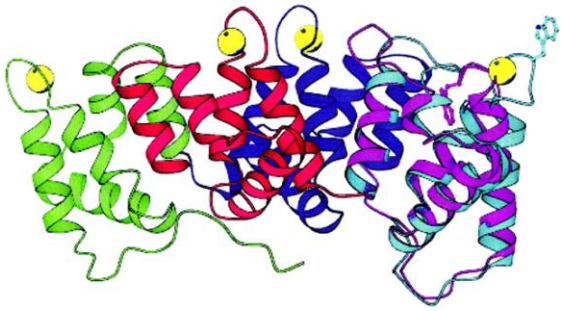

Fig.2.- Crystallized structure showing **"Endonexin Fold"** of conserved 70 amino acid domain sequence in Annexin II.[Kassam G ,1997]

Binding of S100A10 (P11) protein with annexin II reduces the binding affinity of chromaffin granules with annexin II [Jones, 1994]. Distribution of monomeric and tetrameric form differs with the difference of the binding affinity of calcium throughout the cell. It has been reported that annexinII monomer is found in the cytosolic regions (in the cell) and annexin II tetramer is found near the plasma membrane of the cell, usually in calcium ions presence. Calcium ions help in the binding of protein with the plasma membrane. [Jones PG,1994]

Annexin II has a structure having 4 conserved motif domains of 70 amino acids each. These domains are conserved in the protein and most of the amino acids in these motifs are conserved throughout the annexin family. Each motif is composed of 5 α- helices wound into right handed super helix. Within the domain there is a short stretch of amino acids comprising of inter helical loops such that helix-loop-helix is formed. The concave surface if protein faces the cytosol of the cell that contains C-terminal domain and N-terminal domain as well as stabilizes C helix.

Table 1. Ca^{2+}-binding sites of annexin II monomer

Type II sites	
	* * * *
	(1 17) (161)
Domain II	M - K - G - L - G - T - - - - - - - - D
	(201) (246)
Domain III	G - V - K - R- K - G - T - - - - - - E
	(277) (321)
Domain IV	M - K - G - K - G - T - - - - - - - - D
Type III sites	
	* * *
	(49) (52)
Domain I	G - V - D - E -
	* * *
	(87) (95)
	K - S - A - L - - - - E

Fig.3.-**The amino acid sequence of the Ca 2+biding sites of human annexin 2.** The structure of AIIm is composed of 4 domains of similar structure. Each domain contains 5 alpha-helices, denoted A-E, wound into a right-handed superhelix. Within the domain each of the alpha-helices is connected by a short stretch of amino acids comprising an interhelical loop such that two parallel helix- loop-helix substructures are formed (A-loop-B and D-loop- E) and these structures are stabilized by interhelical contacts.[Waisman DM,1990]

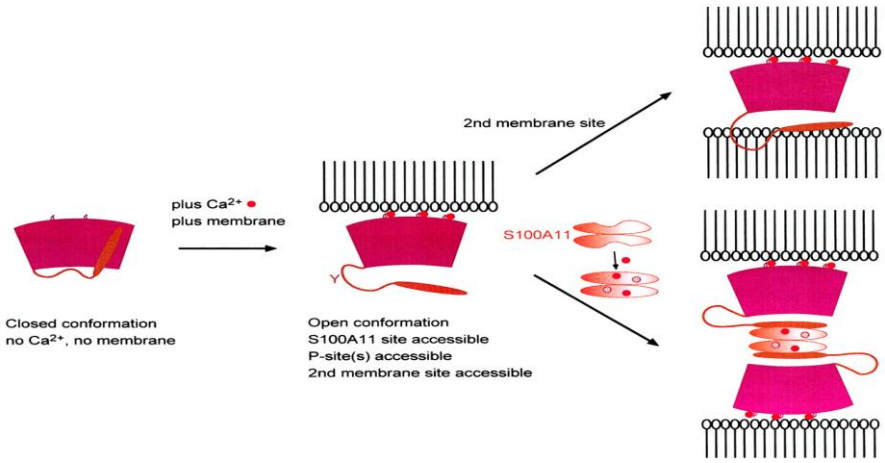

Fig.4.- Binding and bridging of the protein. In the presence of calcium ions, the AIIm gets bound to acidic phospholipids in the plasma membrane which opens the conformation of the protein and S100A10 gets bound to it in a dimeric form which bridges the plasma membrane of two cells.[Lambert *et al*., 2004]

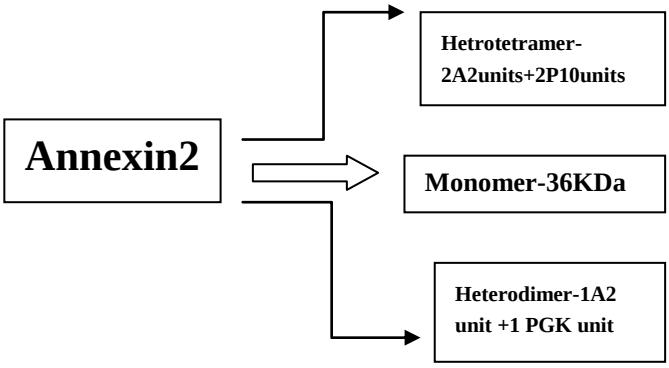

Fig.5.- Annexin 2 (mammalian protein) exists in three forms which have been reported. Annexin 2 is a 36 KDa protein, in its monomeric state, distributed through out the cell. It also exists in hetero dimeric form by binding with one subunit of 3-phospho glycerate Kinase(3-PGK) and in hetero tetrameric form by binding of two monomeric unit of annexin making bond with two P11(11KDa) protein.

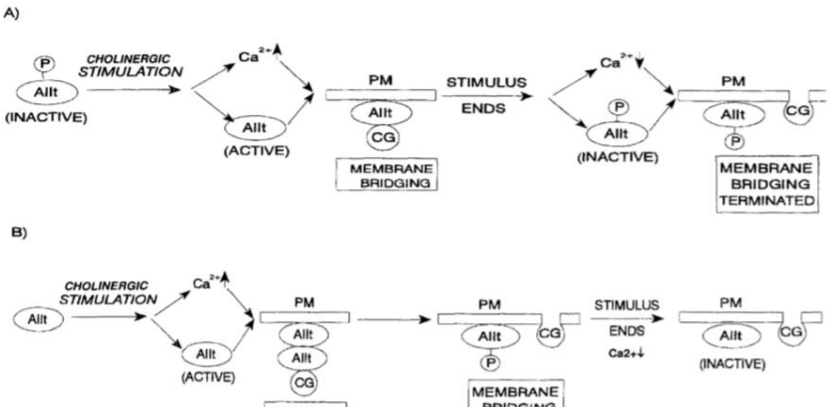

Fig.6.-Regulation of annexin 2 tetramer by serine & tyrosine phosphorylation. Models are proposed to explain how A2t is either phosphorylated or dephosphorylated in presence of chromaffin granules.[Hubaishy *et al*., 1995]
AIIt - Annexin 2 tetramer
PM- Plasma Membrane
CG-Chromaffin Granules

Regulation of annexin II tetramer by serine and tyrosine phosphorylation.

Annexin II monomer contains two major domains- N terminus domain and C terminus domain. N terminus domain has regulatory functions and C terminus has calcium, phospholipid, heparin and P11 binding sites. The first 30 amino acids of c terminal domain in annexin II consists of serine and tyrosine phosphorylation sites. Activation of protein kinase C results in phosphorylation of Serine at 25th position.[2, Waisman DM,1990]. In vitro phosphorylation of the protein at serine 25th position by protein kinase C increases the calcium binding affinity of the protein for phospholipid liposome aggregation which has no effect on phospholipid binding properties of protein . AIIt has also gets phosphorylated in vivo by protein tyrosine phosphorylases. AIIt is also a major in vivo substrate for the constitutive protein tyrosine kinase activity of bovine articular chondrocytes . Activation of growth factor receptors, such as PDGF, has been shown to result in the tyrosine phosphorylation of AII[Jones,1994]. The phosphorylation of AII in pp60 transformed cells or in cells activated by PDGF is identical to the site phosphorylated on the protein in vitro by pp60, namely tyrosine-23. The stimulation of adrenal medulla cells has been shown to result in changes in the phosphorylation pattern of a variety of cellular proteins, and activation of protein phosphatases have been suggested to produce a 48% inhibition of catecholamine release. However, only a single report describes the phosphorylation of AIIt after cholinergic stimulation of cultured adrenal chromaffin cells . Authors reported that AII (monomer and tetramer were immunoprecipitated) was phosphorylated on predominately alkali-sensitive sites (Ser and Thr) during cholincrgic stimulation. Other reports have documented changes in protein kinase activity in adrenal chromaffin cells and reported that upon cholinergic stimulation, the protein kinase C activity is increased and the pp60 Src activity is decreased. Furthermore, it has been shown that inhibition of the activity of calmodulin-dependent protein kinases or protein kinase C are not required for Ca2+dependent secretion, but PKC may play a modulator role.[Jones,1994 & Waisman DM,1990]

Tissue content of annexin II has been reported in avian and mammalian cells. The protein stays absent in heart,smooth muscles, skeletal muscles,liver, platelets, erythrocytes. Low concentratio of protein is reported in brain while intermediate concentrations are reported in adrenal gland, kidney and spleen. Intermediate and high concentrations of annexin II present in different cell types such as epithelial cells of skin, respiratory and intestinal cells, endothelial cells of blood type and chondrocytes of connective and cartilage tissue. Other blood types rich in annexin proteins are fibroblasts, macrophages, splenocytes and HeLa cells.

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Dynamic Reciprocity: role of annexin 2 in tissue integrity

Extracellular Membrane

1.Role of annexin 2 in extracellular matrix (ECM) degradation

2.Annexin 2 mediated cell-cell adhesion [Lambert *et al*., 2004]

Intracellular Matrix

1.Role of annexin2 in membrane organization and trafficking

2.Annexin 2 is entral in cell polarization process

3.Role of annexin2 in endocytosis

4.Annexin 2 and its role in endocytosis mediated cell migration

5.Role of annexin 2 in exocytosis.[5, Waisman DM,1990]

Few differences between monomer and tetramer :-

- Binding affinity of phosphotidylserine to annexin 2 monomer is 2 µM but for tetramer is greater than 10 nM.[Keutzer,1990]
  (Phosphotidylserine, Phosphotidylinositol and phosphatidic acid support high affinity calcium binding but phosphotidylethanolamine and phosphotidylcholine do not.)
- Calcium dependent conformational change includes decrease in α-helix & shift in emission max 342- 334nm. This shift is due to movement of Trp-212 to more hydrophobic environment in presence of calcium. (Trp-212 in monomer is deeply embedded in monomer than in tetramer.)
- Monomeric form is distributed throughout the cell and monomeric form mediate an interaction to form tetramer with the plasma membrane.[Waisman DM,1990]

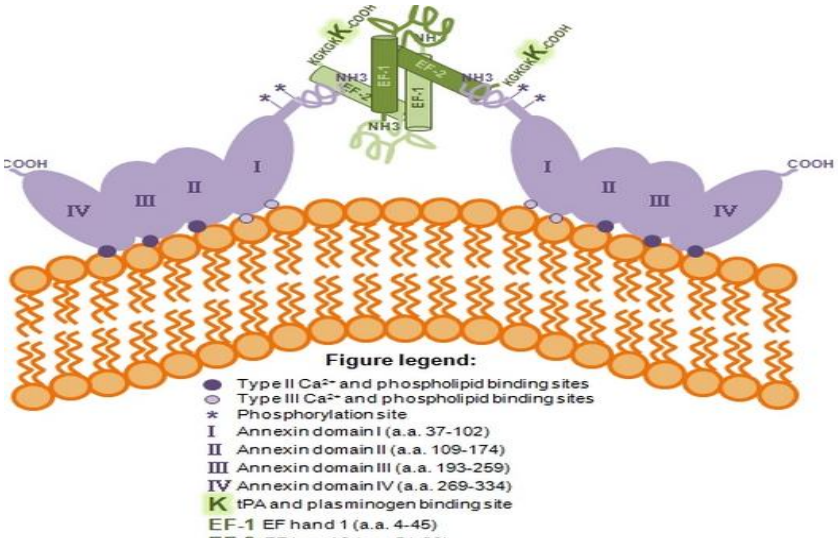


Fig.7.- Annexin 2 tetramer. Annexin 2 tetramer is being formed in this with two annexin units and two S100A10 (P11) units which is further bridging the plasma membrane.

Stimulation of tyrosine phosphorylation of annexin2 by calcium and phospholipids.

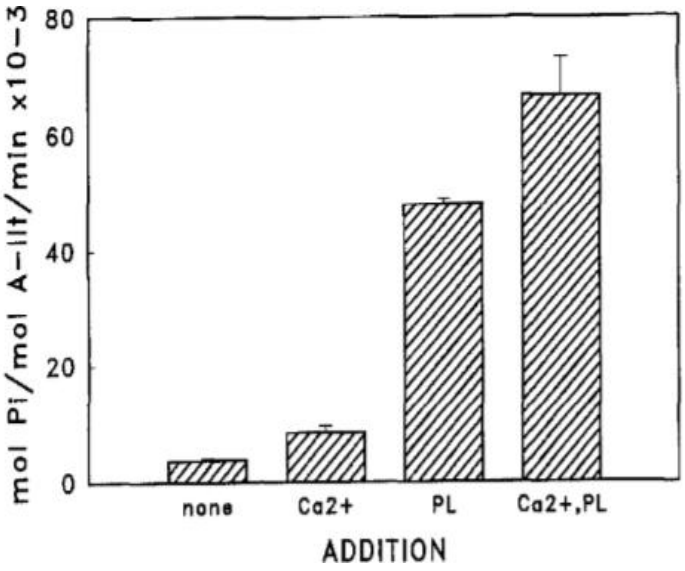


Fig.8.- The initial rates of phosphorylation of AIIt in presence of calcium was about 12% of the rate of phosphorylation of AIIt in presence of both calcium and phospholipids.[Keutzer,1990]

Experimental Procedure Followed :-

Fig.9.-

E.Coli BL21DE3 used for protein expression;

Primary inoculation; grow overnight.

Secondary inoculation and induction (O.D.~0.6-0.8) with 0.6mM IPTG.

Protein preparation. (explained the optimization ahead)

Materials :-

- ***E. coli* Cells**- 1. BL21 DE3
  2. DH5α
  3. Rosetta
  4. BL21DE3 Star
- **Medias Used**- 1. Luria Broth
  2.Terrific Broth
  3. Auto-Induction Media
- **IPTG**- Isopropyl β-D-1-thiogalactopyranoside (Different concentrations)
- **Antibiotics**- 1. Ampicillin
  2.Kanamycin
- **Vectors Used**- 1. pET 11a N-His tag (for Annexin 2)
  2. pBP-N-His tag (for P11)
- Prestained protein ladder (benchmark from Invitrogen)
- Anti-His antibodies (primary and secondary)(Sigma)
- Ponceau staining solution(sigma)
- Substrate & ECL
- Anti P11 antibodies
- Anti A2 antibodies
- Protein G beads

Methods Used :-

1. Competent cells preparation
2. Transformation
3. Plasmid Isolation
4. Induction Optimization
5. Ni-NTA coloumn regeneration
6. Protein Purification
7. SDS-PAGE
8. Degasification & dialysis
9. Desalting & FPLC
10. lyophilization
11. UV spectroscopy
12. Fluorescence Spectroscopy
13. Circular Dichromism
14. Co-Immuno Precipitation
15. Western Blotting

Methods

1. Expression & purification of Annexin II & S100A10

1.1 Competent cells preparation :-

As most of the cells cannot take up DNA directly, they are given chemical and electrical treatment to make them competent, so that they can take up DNA directly. There are two methods for the preparation of competent cells - electroporation and heat shock. Inoculate a single colony in 5ml Luria Broth culture and kept it for over night at 37°C.

1. Used 1ml of the inoculum to re-inoculate in 100 ml Luria Broth and kept it at 37°C for 1.5-3 hours till the O.D. reaches 0.4-0.6.
2. Kept cells on Ice for 20 minutes once removed from incubator.
3. Pelleted down cells at 6000 rpm for 10 minutes at 4°C.
4. Decant the supernatant and gently resuspended in chilled autoclaved solution of calcium chloride and magnesium chloride.
5. Centrifuged cells at 6000 rpm for 10 minutes at 4°C and resuspended the pellet in the same solution. Keep it on ice for 30 minutes.
6. Centrifuged cells at 6000 rpm for 10 minutes at 4°C and discard the supernatant.
7. Resuspended the pellet in 0.1 M calcium chloride +15% glycerol solution.\
8. Dispensed in microtubes (200-300µl/tube).
9. Frozen at -80°C.

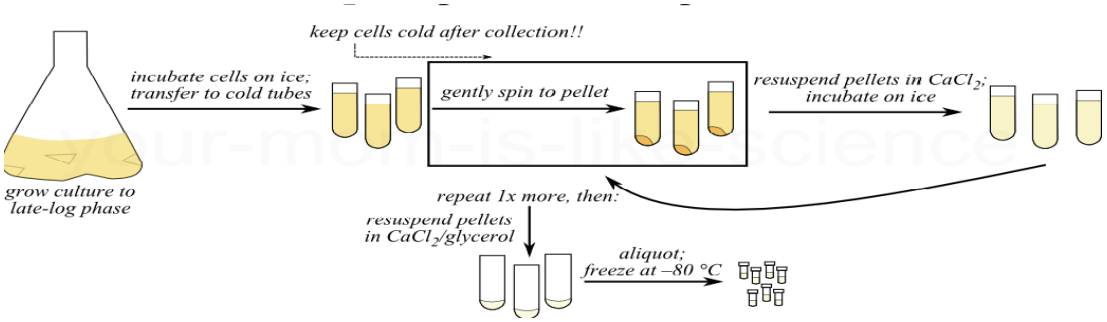


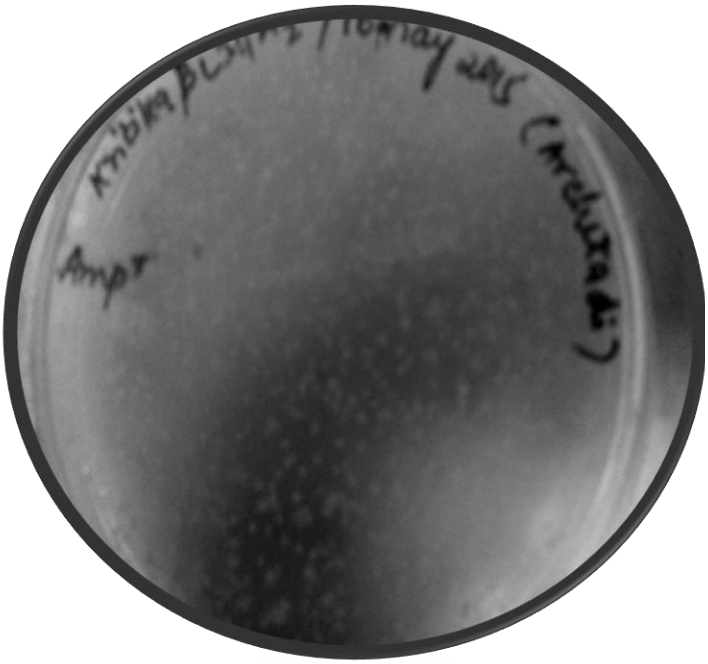
Fig.10.- Flowchart showing protocol followed for the preparation of competent cells using Calcium chloride treatment.

1.2 Transformation :-

Transformation is the direct alteration of cells resulting from direct uptake and incorporation of exogenous genetic material from its surroundings and taken up through cell membrane. Transformation occurs naturally in some species of bacteria, but it can also be affected by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence.

1. Added 2µl of plasmid DNA in 200µl of competent cells.
2. Keep the mixture on ice for 30 minutes.
3. Heat shock of 90 seconds was given to the mixture at 42°C.
4. Kept the mixture on ice for 2-3 minutes.
5. Added 700 ml of LB in the mixture for recovery in the laminar air flow, mix well.
6. Kept the eppendorf at 37°C for 45 minutes.
7. A fast spin for 2 minutes at 140000rpm was given after 45 minutes and 650µl of the supernatant was discarded.
8. The pellet was mixed with the left over supernatant and was plated on LB plate with the appropriate antibiotic marker.
9. Plate was kept at 37°C for overnight.

(A)



(B)

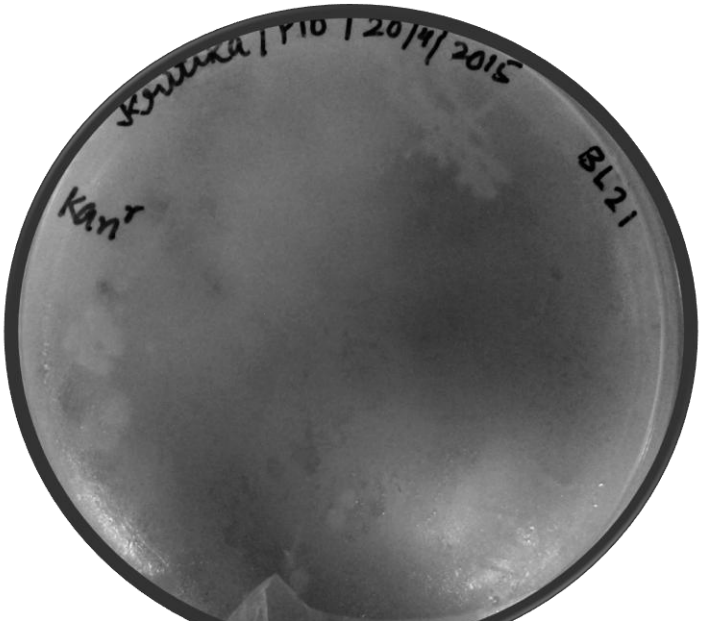


Fig.11. Luria Broth plates of *E.coli* BL21DE3 cells showing (A) plating of annexin II (Amp resistance) and (B) streaking of S100A10 (P11) (Kannamycin resistance).

1.3 Optimization of Annexin 2 isolation from *E.coli* BL21cells

E.coli Bl21 cells are used for protein expression because of the presence of T7 RNA polymerase gene, controlled by the lac UV5 promoter in its chromosomal DNA and the T7 lysozyme gene in the pLys S plasmid. T7 RNA polymerase is expressed upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) which induces a high-level protein expression from T7 promoter driven expression vectors (e.g., pET). The T7 lysozyme suppresses the activity of T7 RNA polymerase2), which reduces the basal level protein expression from the gene of interest.

1. A 30ml primary culture was grown overnight at 37°C.
2. 1% inoculum from the overnight grown culture was added along with 0.1% ampicillin (100 mg/ml) and the flask containing culture was placed at 37°C at 160 rpm.
3. 0.6mM IPTG induction was given as the O.D. reaches 0.6-0.8 (approximately after1.5-2hours).
4. Culture was left for 5 hours on 37°C at 180 rpm.
5. Grown culture's cells were pelleted down at 4°C for 15 minutes at 5000rpm.
6. Supernatant was discarded and the pellet was dissolved in 5 ml of the lysis buffer.
7. 0.1mM PMSF was added to the dissolved pellet and the sample was sonicated at 32% amplitude thrice for 3 minutes each, till it turns brown from cream color.
8. Sonicated samples was transferred to oakridge tubes and samples were placed in ice.
9. The samples were centrifuged again for 60 minutes at 14000 rpm at 4°C.
10. The supernatant was transferred to fresh or autoclaved flacons and Ni-NTA beads were added in it. The falcon was placed in 4°C on the rocker for 2 hours for binding.
11. A short spin at 4°C was given to settle the beads and flow through was collected.
12. Washes of 10mM (50ml) and 20 mM (50 ml)were given and eluted with 250 mM.
13. Ran a 12% SDS PAGE gel and pooled the pure single band proteins in a fresh falcon.
14. Dialyzed the protein in phosphate buffer with pH 8.0 for over night.
15. Stored the dialyzed protein in a fresh falcon at 80°C.

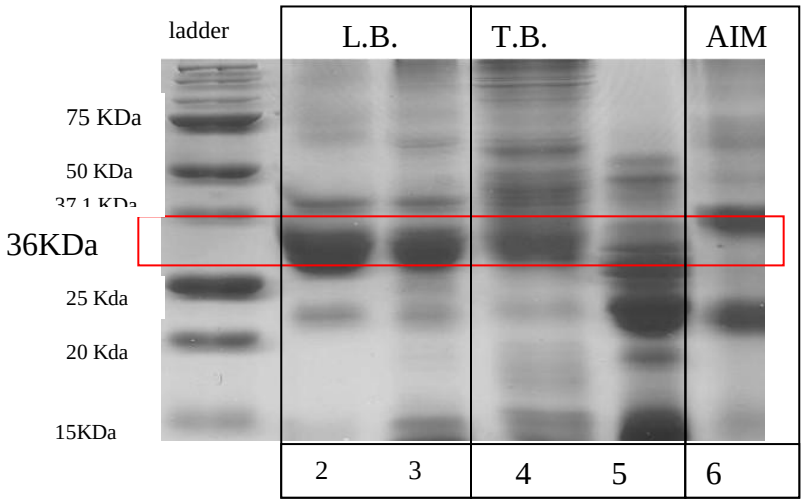


Fig.12.- A 12% SDS-PAGE gel was run to check the induction in annexin II with LB media, TB media and AIM. Well 1 contains Protein marker. Well 2 and 3 contains cells grown in LB media and induced with 0.6mM and 0.8mM IPTG respectively. Well 4 and 5 contains cells grown in TB media and induced at 0.6mM and 0.8mM respectively. Well 6 contains cells grown in AIM.

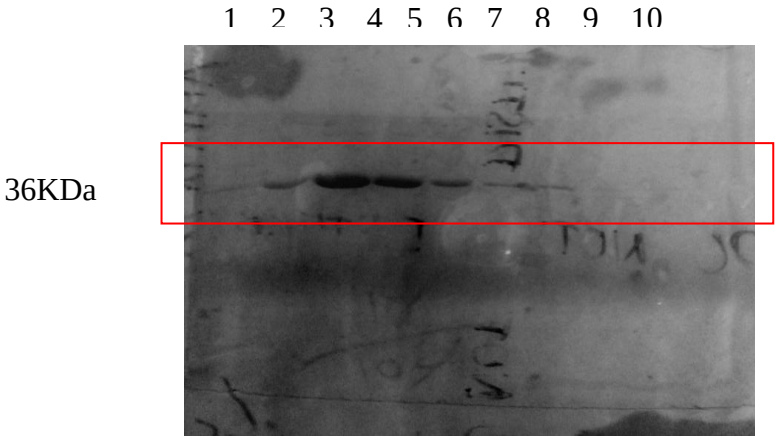


Fig.13.- A 12% SDS-PAGE gel was run to check the purity of the protein. Well 1 contains 30 mM imidazole wash fraction and Well 2 to 9 contains 250mM imidazole eluted fractions.

1.4 Optimization of Isolation of P11 from *E.coli* BL21 cells.

E.coli Bl21 cells are used for protein expression because of the presence of T7 RNA polymerase gene, controlled by the lac UV5 promoter in its chromosomal DNA and the T7 lysozyme gene in the pLys S plasmid. T7 RNA polymerase is expressed upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) which induces a high-level protein expression from T7 promoter driven expression vectors (e.g., pET). The T7 lysozyme suppresses the activity of T7 RNA polymerase2), which reduces the basal level protein expression from the gene of interest.

1. A 30 ml primary was inoculated in Luria Broth at 37°C for over night.
2. 1% of this primary culture was inoculated along with 0.1% kannamycin antibiotic resistance in terrific broth culture and the culture was kept at 37°C.
3. 0.6mM IPTG induction was given to the culture when the O.D. had reached around 0.6-0.8 and the temperature was changed to 20°C for over night.
4. Cells were pelleted down at 5000 rpm at 4°C for 15 minutes.
5. Supernatant was discarded and the pellet was resuspended in 5ml lysis buffer and Protease cocktail inhibitor was added (0.1mM).
6. The sample was sonicated at 32% amplitude twice for 3 minutes and once for 1 minute till the color shifts from cream to light brown and density decreases.
7. Sonicated samples were centrifuged at 14000 rpm for 60 minutes at 4°C in oakridge tubes.
8. Supernatant was transferred to a fresh or autoclaved falcon and Ni-NTA beads were added to the supernatant. Pellet was discarded.
9. Mixture was kept on rocker for 2 hours at 4°C for binding.
10. A short spin was given to the sample at 4°C only and the flow through was collected, beads were transferred to the column.
11. Washes of 10mM(100ml), 30mM (30ml) and 50mM (10 ml) were given and the protein was eluted at 250mM.
12. Fractions were collected in eppendorfs and an SDS PAGE 15% gel was run.
13. Pure fractions were pooled together and lyophilized.

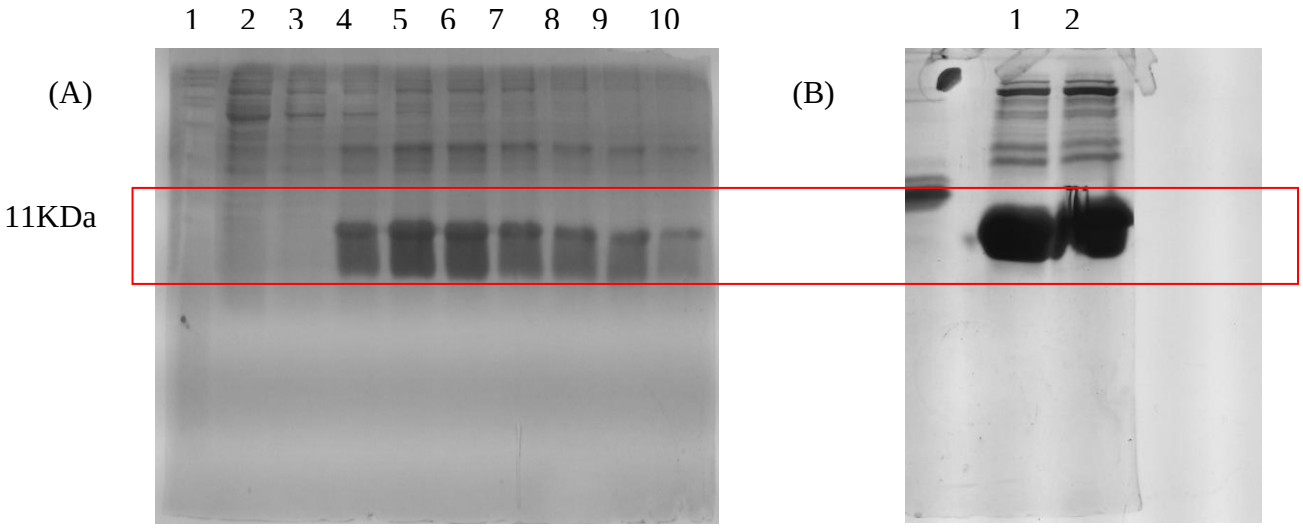


Fig.14.- 15% SDS-PAGE gel was run to check the purity of S100A10 (P11) protein.

Gel (A) Well 1- Ladder, Well 2& 3- 50mM imidazole wash fractions and Well 4 to 10- 250 mM imidazole elution fractions of P11 protein.

Gel(B) Well 1 & 2- Purified fractions of P11 protein, eluted at 250 mM imidazole.

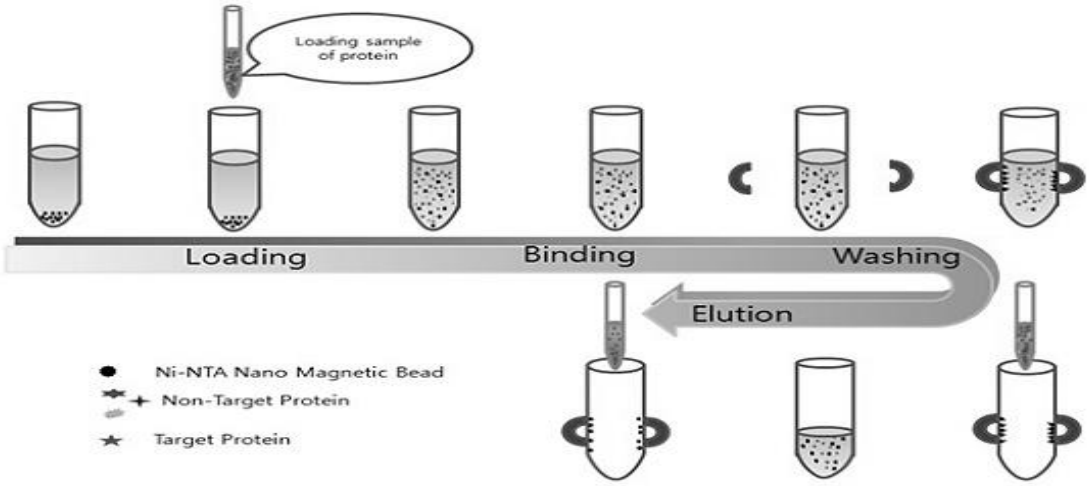


Fig.15.- An image showing steps for isolation of protein from cell lysate.

1.5 Plasmid Isolation from *E.coli* DH5α cells.

Plasmid isolation is a method of isolating DNA from bacterial cells. Usually DH5α cells are used for plasmid isolation because this strain has the ability to accept plasmid insertion exceptionally well. This strain was developed by D. Hanahan as a cloning strain with multiple mutations that enable high-efficiency transformations. The mutations that the DH5-Alpha strain has are: dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1. These mutations correspond to the distinct characteristics that make the DH5-Alpha strain excel in laboratory cloning procedures. lacZ Delta M15 mutation: Allows for blue-white screening for recombinant cells. (Amnion Kits were used.)

1. A colony from DH5α plate was inoculated in 30 ml LB media containing the antibiotic resistance marker and was kept at 37°C for overnight to grow.
2. Culture was centrifuged in a fresh falcon for 20 minutes at 3500 rpm at 4°C.
3. Supernatant was discarded and the pellet was resuspended in 250µl plasmid resuspension buffer containing RNAase.
4. The solution was transferred to a fresh eppendorf and 250µl lysis buffer was added and eppendorf was inverted 3-4 times.
5. Immediately 350µl of neutralization buffer was added and eppendorf was invert 3-4 times.
6. Centrifuged the suspension solution at 14000rpm for 40 minutes to settle the pellet completely.
7. Removed the supernatant to a fresh eppendorf without touching the pellet and add equal amount of isopropanol.
8. Invert the mixture 3-4 times and tranfer 600µl of the solution to a fresh spin column.
9. Short spin was given to the column and the collection tube converter was discarded and remaining solution was again transferred to the spin column and a short spin was given.
10. 500µl of plasmid clean up buffer was added and was given a spin.
11. Wash buffer with ethanol was added and a short spin was given again.
12. Column was left for 20 minutes for ethanol to get evaporated.
13. 50µl of Milli-Q water was added and the collection tube was replaced by a fresh collection tube before giving a short spin.
14. O.D. was checked on a nanodrop and 0.8% agarose gel was run to check the purity of the samples.

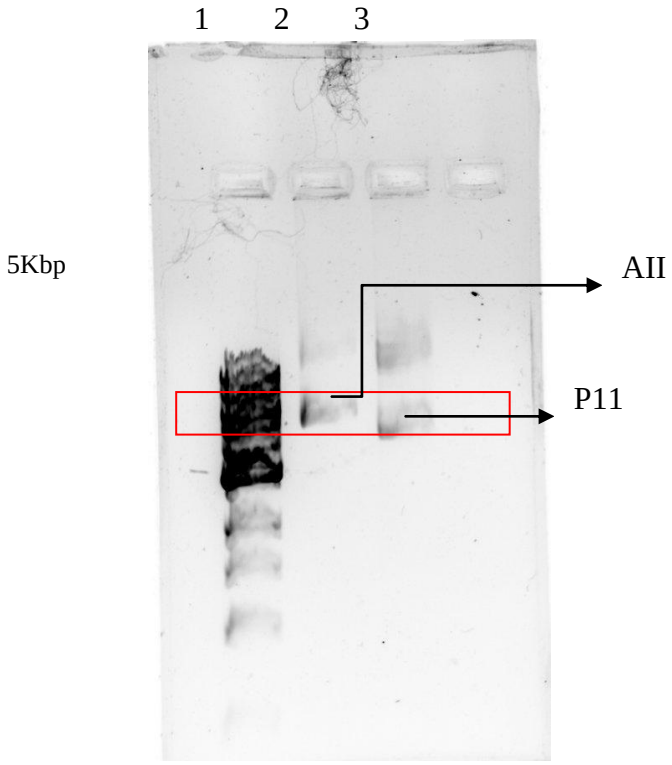


Fig.16.- 0.8% agarose gel showing plasmid run. Well 1 contains DNA marker. Well 2 contains pET 11a N-His tag (Annexin II's plasmid) with concentration of 90 ng/µl. Well 3 contains pBP N-His tag (S100A10's plasmid) with a concentration of 80 ng/µl.

Results

2.1 Sequencing of Annexin II and S100A10(P11) purified proteins.

Protein Sequencing is a technique to determine the amino acid sequence of a protein which further gets useful in studying about the folded structure of proteins. Major methods for protein sequencing are : 1. Mass Spectroscopy 2. Edman Degradation.

It is also possible to generate amino acid sequence with the DNA/RNA sequence available.

Annexin II and S100A10 (P11) sequencing results were performed by amnion and the concentration of the samples were 50 µg/ml. Annexin II has hexa histidine tag at its C-terminal and S100A10 (P11) has hexa histidine tag at its N-terminal.

(a) Sequence analysis of S100A10 (P11) protein

HHHHHHMPSQMEHAMETMMFTFHKFAGDKGYLTKEDLRVLMEKEFP GFLENQKDPLAVDKIMKDPDQCRDGKVGFQSFFSLIAGLTIACNDYFVVH MKQKGKK

(b) Sequence analysis of Annexin II protein

GTVHEILCKLSLEGDHSTPPSAYGSVKAYTNFDAERDALNIETAIKTKGVD EVTIVNILTNRSNQRQDIAFAYQRRTKKELASALKSALSGHLETVILGLLK TPAQYDASELKASMKGLGTDEDSLIEIICSRTNQELQEINRVYKEMYKTD LEKDIISDTSGDFRKLMVALAKGRRAEDGSVIDYELIDQDARDLYDAGVK RKGTDVPKWISIMTEVPHLQKVFDRYKSYSPYDMLESIRKEVKGDLENAF LNLVQCIQNKPLYFADRLYDSMKGKGTRDKVLIRIMVSRSEVDMLKIRSE FKRKYGKSLYYYIQQDTKGDYQKALLYLCGGDDHHHHH

2.2 UV Sprectal analysis of Annexin II and S100A10 (P11)

Elextronic spectra involves transitions between different energy levels of the elctrons in a molecule. Electromagnetic radiation causes electrons to oscillate in response to incident electric feild. Electromagnetic transitions give rise to the familiar manifestations of the interaction of electromagnetic radiation with matter: color, vision and conversion of sunlight into plants. The energies involved in electronic transitions correspond to the absorption of photons in visible (400-750 nm) and ultravoilet (200-400 nm) regions of electromagnetic spectrum. Below 200 nm is far-or-vaccum- ultra voilet region, so called because absorption by oxygen of the air is considerable in this region and spectra can be obtained only if the spectrometer is evacuated.

The most general application of electronic spectroscopy is to measure concentrations- for example of proteins, NADH and nucleic acids because in most of the cases there is a direct correlation between number of molecules present and absorption.

1. 100µl of pure dialyzed fractions were taken in a fresh eppendorf.
2. Blank was set with PBS pH7.4 and cuvette was washed with RO water.
3. Protein was diluted to four times with PBS pH 7.4
4. Readings were taken and graph was plotted.

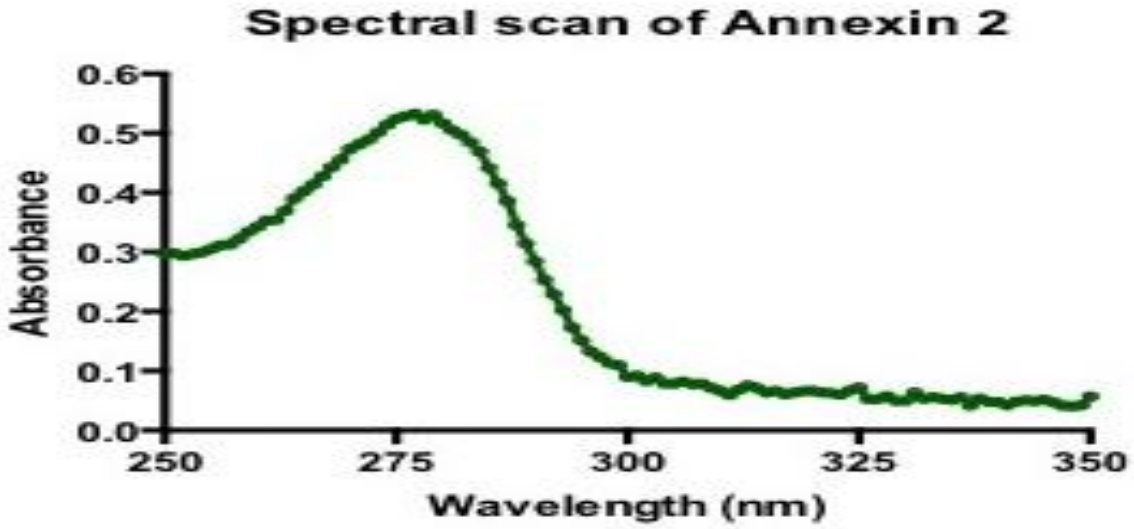


Fig.17.- Graphs showing UV spectra for annexin II.

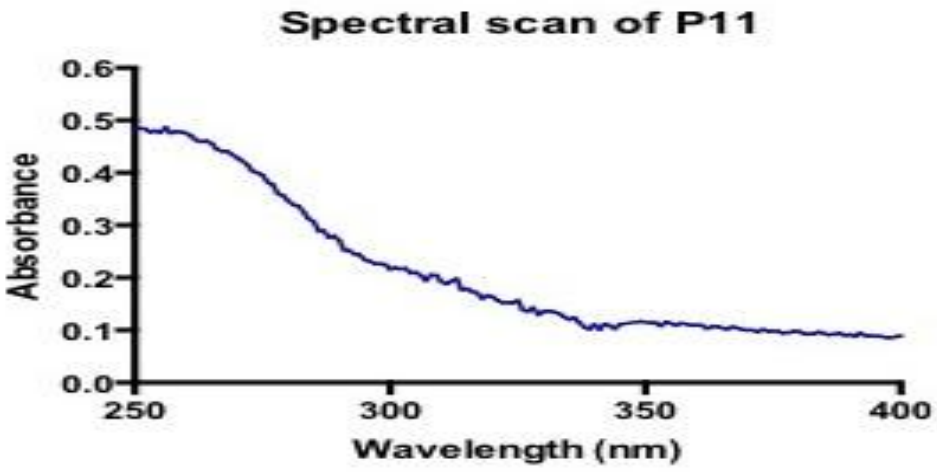


Fig.18.- Graph representing UV spectral analysis for purified P11 protein.

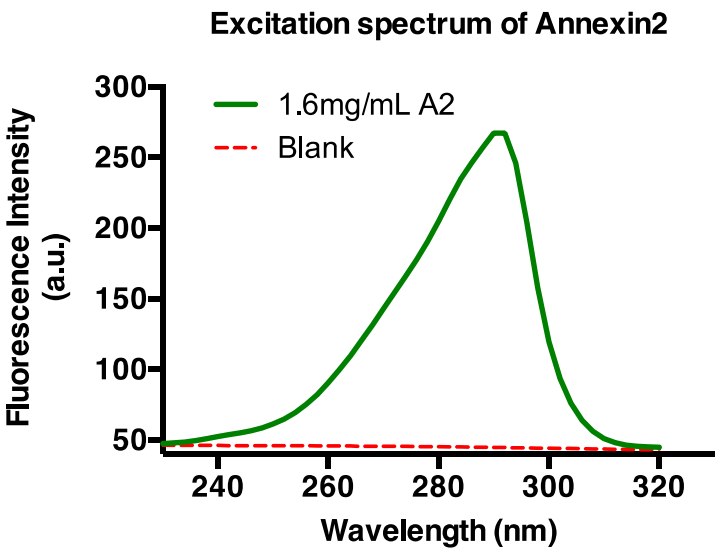
2.2 Fluorescence Spectroscopy for Annexin 2 and P11 proteins.

Fluorescence involves two processes : Absorption & subsequent emission. Each process occurs in a time scale given by the inverse of transition frequency but there is a time lag of about 10^{-9} sec when the molecule exists in the excited state. The lifetime of the molecule in the excited state depends on the competition between the radiative emission and any radiation less process, such as transfer of excitation energy to the surrounding medium. These non radioactive processes provide an alternative mechanism for excited molecules to return back to the ground state, and their presence will result in a diminution or a quenching of fluorescence intensity.

The fluorescence spectrum of a protein can be used to assess the folded tertiary structure of the protein and a comparison between the previously known data and the data obtained can be used to confirm the foldedness of the purified protein.

1. 200µl of each protein were taken.
2. Blank was run as PBS pH7.4.
3. Excitation spectra of each protein was measured from 220-320nm.
4. Emission spectra of each of them was measured from 300-400nm.
5. Graph was plotted and results were interpreted.

(A)



(B)

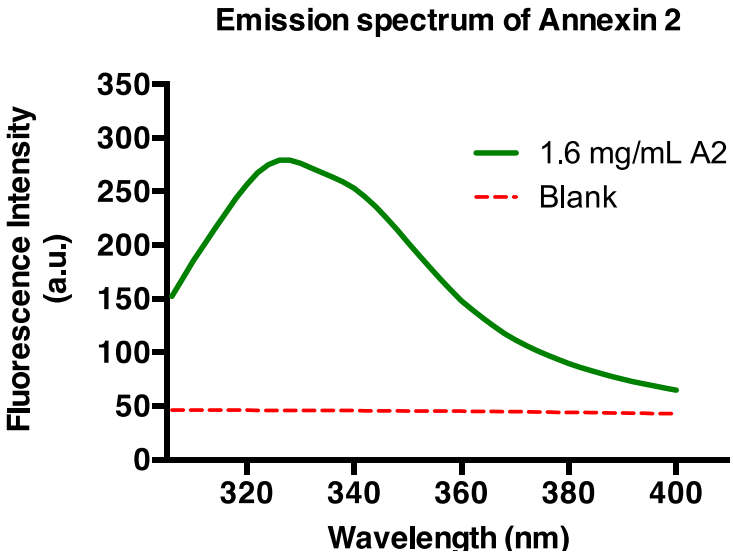
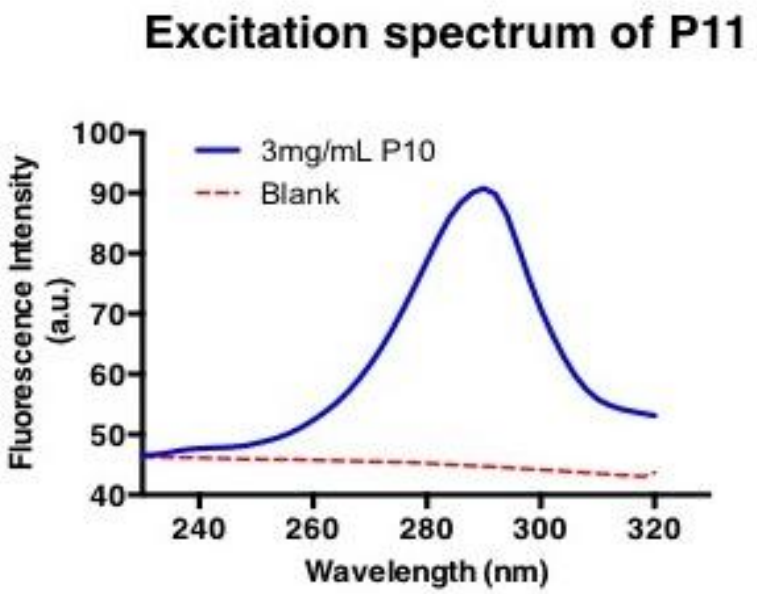


Fig.19- Graphs representing fluorescence spectra of Annexin II.

Graph (A) shows excitation spectra of AII(1.6 mg/ml) which was performed from 220-320nm.

Graph (B) shows emission spectra of AII(1.6 mg/ml) which was performed from 300-400nm.

(A)



(B)

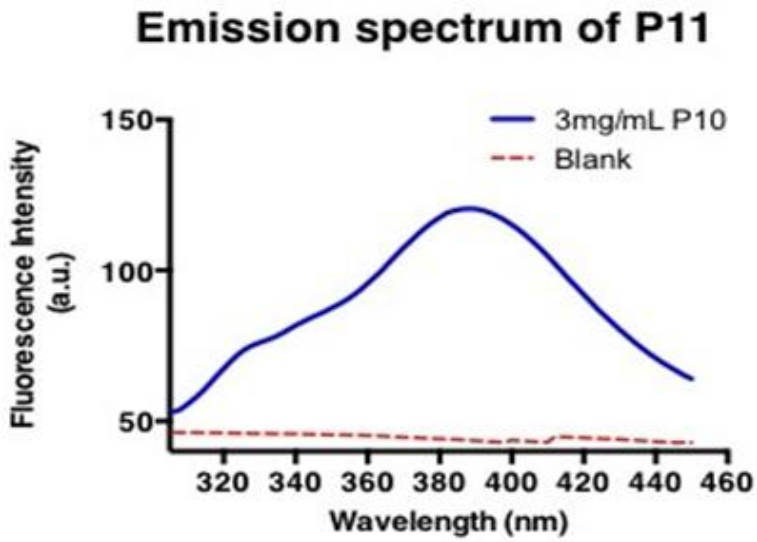


Fig.22.- Graphs representing fluorescence data of S100A10 (P 11).

Graph (A) represents excitation spectra from 220-320nm of P11 (3 mg/ml).

Graph (B) represents emission spectra from 300-400nm of P11 (3 mg/ml).

2.3 Circular Dichroism of Annexin 2.

CD arises from the differential absorption of **L** and **R** polarized light. Thus, it could be detected by a double beam instrument that puts **L** in one beam and **R** in the other. CD can be used to determine the secondary structure of proteins. The approach was laregely empirical and was absed on the CD spectra of the peptide backbone (190-250nm) for model polypeptides. The structure of these models was known from X-ray data and it was assumed that in solution these polypeptides had only a single conformation. For proteins, the main standards were the three forms of poly-L-lysine : α- helix, β-sheet and random coil (R). The CD spectrum of the protein was then simulated by a suitable graphical addition of three curves.

CD spectra was performed and analysis of A2 to check for the structural integrity of the purifies protein.

1. 100µl of dialyzed sample was put into the cuvette.
2. Blank was set with PBS pH 7.4 and cuvette was cleaned with RO water.
3. Spectra was measured from 250 nm to 200 nm.
4. Graph was plotted and results were interpreted.

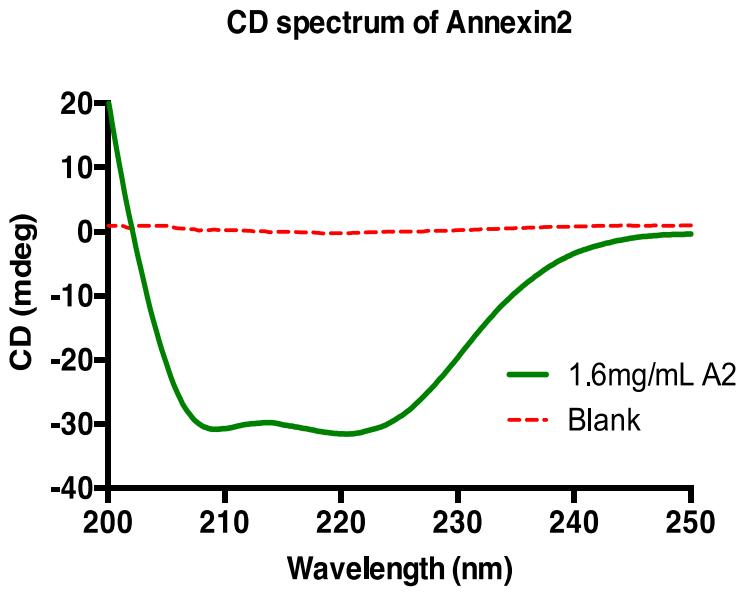


Fig.21.- Graph representing CD spectra of AII (1.6 mg/ml) from 250 nm to 200 nm.

2.4 Co-Immuno Precipitation of Annexin 2 and P11 proteins.

Co-IP has the fundamental principle of the specific antigen-antibody reaction. Co-IP helps determine whether two proteins interact or not in physiological conditions in *vitro*. The known protein (antigen) is termed the bait protein, and the protein it interacts with is called the prey protein. After the cells are lysed under non denaturing condition proteins that bind together are kept. Therefore one can use anti-P to bind with P and precipitate it through Co-IP.

Co-IP is used to find whether the bound protein interacts with the known protein or not and to find new proteins which bind to the known protein.

Advantage- Proteins that give positive results for Co-IP are post translationally modified and are in their stable conformation.

Disadvantage- Proteins with low signals may not be detected or there can be a possibility of third protein being involved in the interaction to get stablized, which may may not interact.

Co-IP is not just restricted to cell lysate to study interactions between proteins but is also useful to study interactions when proteins are in their pure states and also helps us to study the binding ratio of proteins.

1. 100µl of annexin 2 (10 mg/ml) concentration and 300µl of P11 (3mg/ml) was put together in the same eppendorf for 2 hours. Mixture was placed at 4°C in the rocker.
2. 1µg antibody against P11 protein(mouse raised) was added to the solution. Solution was kept on the rocker at 4°C for over night for binding to take place.
3. 30µl of washed protein G beads were added to the solution and were allowed to bind with antibodies for 2.5 hours at 4°C.
4. A short spin at 4°C was given to the solution , supernatant was collected in a fresh tube and three four washes were given to the beads with PBS pH 7.4, which had settled down like a pellet, for excess protein or unwanted proteins that were weakly bonded with the antibodies on the beads.
5. One 15% SDS PAGE gel was run, with beads bound protein complex as one sample and supernatant as other sample and dye front was allowed to come out of the gel in the running buffer.
6. The gel was placed in the western transfer cassette and proteins were transferred to the nitrocellulose membrane with the apparatus placed in ice.
7. After transfer the blot was removed and kept in 5% BSA for blocking for 2 hours.
8. A short 5 minutes wash was given to the blot and A2 primary antibodies (1:1000 rabbit raised) in 5% BSA were added to the blot and it was kept for binding overnight at 4°C.
9. Four 15 minutes washes were given with TBST at room temperature and secondary antibodies (1:20,000) were added on the blot and was kept for 1 hour at room temperature on the rocker.
10. Four 15 minutes washes were given to the blot and the blot was developed with the help of ECL substrate.
11. Blot was viewed under gel doc.
12. The membrane was stripped and antibody for P11(1:1000 mouse raised) was added in 5% BSA at 4°C for over night.
13. Blot was developed in the similar manner and was viewed on gel doc.

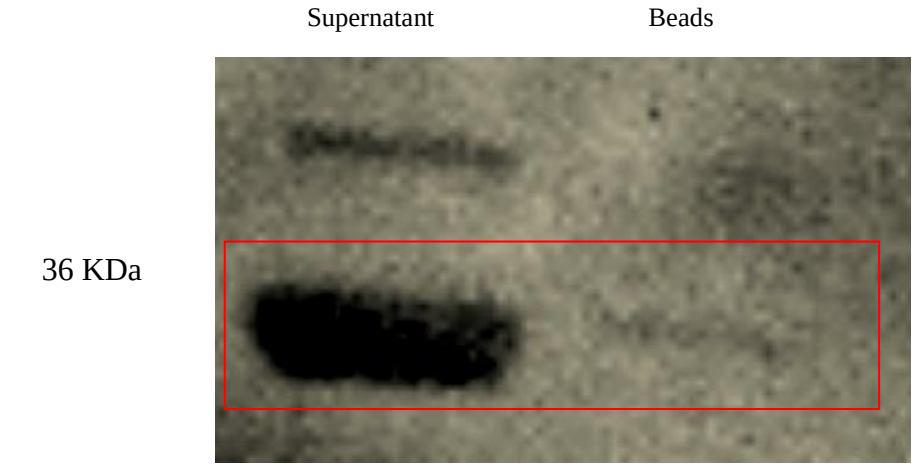


Fig.22.- Western blot of Co-immuno precipitation with antibody against Annexin 2(rabbit raise) protein. The first well was loaded with invitrogen benchmark prestained ladder, second well was loaded with supernatant and third well was loaded with beads. Presence of A2 in third well proves that P11 binds with A2 and forms a functional complex.

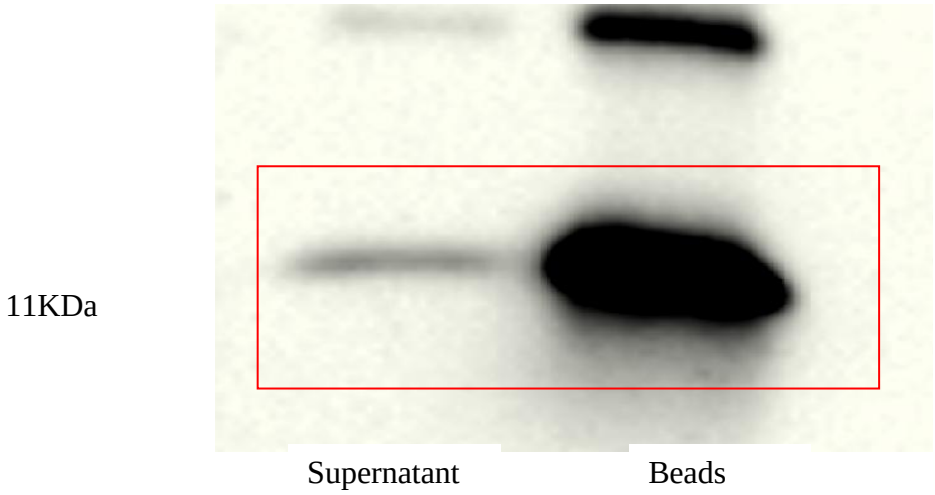


Fig.23.- Western blot of Co-immuno precipitation with antibodies against P11 protein(mouse raised) were added. The same blot was stripped and bands were observed. Thick band in beads signify more amount of P11 was trapped by the antibodies Of P11 protein and very less of it was remaining in the supernatant. This step was performed for cross verification of presence of P11 protein in supernatant and beads.

Discussion

Cloning and purification of Annexin II and P11 proteinswas done *E.coli* cells. The objective here is to characterize the interactome of annexin II. To start with the interaction of annexin II and P11 was to be established. The construct comprise of pET 11a N-His tag vector and pBP N-His tag vector for annexin II and P11 respectively. Proteins were purified using Nickel -NTA resins by affinity chromatography. The proteins were eluted at 250mM imidazole ionic strength fractions. The pure fractions of annexin II were dialyzed using phosphate buffer (20mM) pH 8.0 and pure fractions of P11 were desalted on FPLC chamber using desalting colomn.

Optimization of annexin II was performed using different medias- Luria broth, terrific broth and auto induction media. Induction check at 0.6-0.8mM iptg was performed in cell lysate. Luria broth showed the best results at 0.6mM IPTG concentrations.

Optimization of P11 was also performed using different medias- Luria broth, Terrific broth and auto induction media. Terrific broth was showed best results at 0.6mM IPTG concentration.

E.coli BL21 cells were used in each case for over expression of proteins and DH5α cells were used for plasmid isolation.

UV spectral analysis gave the concentration of the protein. It also showed the absorbance spectra of the proteins which was helpful in determining the wavelength necessary for excitation spectrum for fluorescence studies.[Dialyzed samples were used for all the spectral analysis.]

Fluorescence studies were conducted to check the tertiary structural integrity of proteins and the data thus obtained showed that annexin II was having a well folded tertiary structure, well coinciding with the previously published data.

Emission and excitation fluorescence spectra was conducted to check the stability of the protein. Peak at 295nm in excitation spectra shows presence of tryptophan moiety in the protein sequence.

Emission Spectra showing differences in fluorescence intensities helped us to conclude, that tryptophan moiety in annexin II might not be exposed to the hydrophilic surface and In P11 tryptophan moiety might be exposed to the hydrophilic surroundings.

To check whether the proteins have maintained their structural integrity , Circular dichroism was performed.

Annexin II's Circular Dichroism's spectra showed peaks at 222nm, 208nm and 217nm which is due to the presence of α-helices and β-sheets. From CD spectra the K2D2 software predicted α-helix content as 84.72% and β-strand content as 1.24%.

Lastly, co-immuno precipitation was performed to see the interaction between annexin II and P11. Western blot was performed and presence of annexin II in third well, where G beads were loaded proves that P11 binds with annexin II and forms a functional complex. The blot was stripped & checked with antibodies also, to see the presence of P11 proteins.

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