MATERIALS AND METHODS
2.0 MATERIALS AND METHODS

2.1 MATERIALS.

2,4,6,7-[3H] estradiol 17β (sp.act, 101 Ci/mmol) was obtained from Amersham. Monoclonal anti-hsp25 (clone IAP-28), monoclonal anti-hsp 70 (clone BRM-22) and anti-actin antibodies, 5-Bromo 4-chloro 3-indolyl phosphate (BCIP), Nitroblue tetrazolium (NBT), Fluorescein isothiocyanate (FITC) isomer I, estradiol 17β, Triton X-100, Nonidet P-40, Quercetin, CM-Sephadex, DEAE-Sepharose, protein A-Sepharose, 4-chloro-1-naphthol, unprocessed wheat germ and the dialysis bags were obtained from Sigma chemical Co. Sepharose 4B, Sephadex G-25 and Sephadex G-100 were purchased from Pharmacia. DEAE cellulose was obtained from Whatman. Nitrocellulose membranes were purchased from Schleicher and Schuell Inc., USA. Concanavalin A and HRPO (Horse radish peroxidase) or alkaline phosphatase coupled goat anti-rabbit IgG and alkaline phosphatase coupled rabbit anti-mouse IgG were purchased from Genei, Bangalore. Phenyl methyl sulphonyl fluoride and silver nitrate were purchased from E-Merck, Germany. Estrogen receptor NLS peptide (amino acid sequence from 256-303) and HBD peptide of ER (aminoacid sequence from 302-320) were synthesized at the Rajiv Gandhi Center for Biotechnology, Trivandrum. Routine chemicals used were of the analytical grade and were purchased from local commercial establishments.

Distilled water used in these studies: The raw water was first subjected to pressure filtration in order to remove suspended particles. This was then deionized with the help of a commercial (Purewater Systems) de-ionizer. The deionized
water was subjected to single distillation in a glass distillation unit. This water was re-distilled using a quartz-glass distillation unit.

2.2 BUFFERS.

1) TEM buffer: 50 mM Tris-HCl pH 7.6
   1 mM EDTA,
   12 mM Monothioglycerol,
   0.2 mM PMSF

2) TEMN buffer: 50 mM Tris-HCl pH 7.6,
   1 mM EDTA,
   12 mM Monothioglycerol,
   50 mM NaCl,
   0.2 mM PMSF.

3) TCKM buffer: 50 mM Tris-HCl pH 7.6,
   1 mM CaCl₂,
   20 mM KCl
   2 mM MgCl₂,
   0.2 mM PMSF.

TCKM buffer was made as a 5X buffer and stored at 4°C. It was diluted to X and the following buffers were made for the isolation of nuclei.

Buffer A: TCKM (X)+ 250 mM sucrose.
Buffer B: TCKM (X)+ 250 mM sucrose+0.05% Triton X 100.
Buffer C: TCKM (X)+ 340 mM sucrose.

4. Nuclear transport

 assay buffer: 50 mM Tris-HCl pH 7.6,
   3 mM CaCl₂,
   5 mM MgCl₂,
   25 mM KCl,
   500 mM Sucrose,
   4 mM ATP.

5) TMC buffer: 2 mM Tris-HCl pH 8.0,
   0.5 mM β-mercaptoethanol,
   0.2 mM CaCl₂.
6) **Imidazole buffer:** 10 mM Imidazole pH 8.0, 0.5 mM β-mercaptoethanol, 0.1 mM CaCl₂, 1 mM ATP.

7) **PM buffer:** 10 mM Sodium phosphate pH 6.5, 10 mM MgCl₂.

8) **Towbin buffer:** 25 mM Tris-HCl pH 8.3, 192 mM Glycine, 20% methanol.

9) **TNN buffer:** 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% NP-40.

10) **TMg buffer:** 10 mM Tris-HCl, pH 7.6, 6 mM MgCl₂.

11) **ScintillationCocktail:** :5 g PPO, 500 mg POPOP in Toluene:Triton X-100 (67:33 V/V).

2.3 PREPARATION OF GOAT UTERINE CYTOSOL.

Goat uteri were obtained from a local slaughter house, transported on ice to the laboratory and kept frozen at -75°C until further use. Goat uterine cytosol was prepared following the procedure of van der Hoeven (1981). The uteri were homogenized in the cold using a polytron (PT-45-80) homogenizer at setting 4 for 30 seconds (× 4) with sufficient time in between to prevent heating of the homogenate. The homogenate was filtered through a nylon mesh. The filtrate was centrifuged at 10,000 X g for 30 minutes. The fat layer was carefully aspirated off and the supernatant was filtered through glass wool. Polyethylene glycol (PEG) 6000 was added to the filtrate to a final 5% concentration. The filtrate was stirred in the cold for 30 minutes. The stirring was monitored at a specific speed in order
to avoid frothing of the filtrate. The microsomes which were precipitated with 5% PEG were pelleted at 10,000 X g for 30 minutes. The post-mitochondrial supernatant (cytosol) was filtered again through the glass wool.

2.4 ISOLATION OF GOAT UTERINE NUCLEI:

Goat uterine nuclei were isolated following the procedure developed by Thampan (1985). Goat uteri were obtained from the slaughter house, transported on ice to the laboratory and used immediately for the isolation of nuclei. The uteri were cleaned off the adhering fat tissue and was minced very finely. A 20% homogenate was made in buffer A using a Polytron homogenizer at setting 4 for 30 seconds four times with sufficient time in between to prevent heating of the homogenate. The homogenate was filtered through a fine nylon mesh and through a nylon membrane of 80-120 microns. The filtrate was then centrifuged at 800 X g for 10 minutes. The nuclear pellet was washed with the buffer B followed by two washes with the buffer A to remove Triton X-100. The nuclei were suspended in buffer A and 5 ml of this suspension was layered over 30 ml buffer B and centrifuged at 800 X g for 10 minutes. The final pellet contained purified nuclei with 80% yield as observed under a phase contrast microscope.

To obtain nuclei with the nuclear membranes intact, the above procedure was followed except that the nuclei were not treated with the buffer B which contained 0.05% Triton X 100 which solubilizes the nuclear membranes.

2.5 NUCLEAR TRANSPORT ASSAY FOR ER TRANSPORT INTO THE NUCLEUS.

The nuclei to be used in the transport assay were isolated with their membranes intact or without membranes as mentioned in the previous section. The
nuclei were suspended in 2X assay buffer. The ER was isolated following the procedure described by Zafar and Thampan (1993) (This procedure is detailed in the section 2.10)

100 μl of the ER preparation was incubated overnight with 20 nM $^3\text{H}$-estradiol (50μl). The unbound hormone was removed following adsorption of free hormone to dextran coated charcoal (100μl) (1% charcoal and 0.1% dextran in TEMN buffer). 200μl of $^3\text{H}$-E$_2$-ER complexes (containing ~2μg) were incubated with goat uterine nuclei (250 μl) in the presence of 50 μl aliquot of the fractions containing the p55 for 30 minutes at 30°C in a water bath. The incubation was terminated by transferring the assay tubes to an ice bath and dilution of the incubation mixture by the addition of 2 ml ice cold assay buffer. The nuclei were sedimented at 5,000 X g for 2 minutes. The nuclei were washed once again with 2 ml assay buffer and finally the radioactive hormone associated with the nuclei was extracted with 1 ml distilled ethanol. The ethanol extracts were transferred to glass vials containing 10 ml scintillation cocktail. The radioactivity was measured using a Wallac liquid scintillation counter.

2.6 FLUORESCENCE ASSAY OF THE ER TRANSPORT INTO THE NUCLEUS:

ER was labeled with FITC as described in section 2.15. Goat uterine nuclei were isolated as described above and were suspended in the nuclear transport assay buffer. The nuclei were incubated with FITC-ER (5μg) in the presence or absence of p55, p28 and p75 at 30°C. Aliquots of the incubation mixture were transferred to a glass slide and covered with a glass coverslip. The transport of ER
into the nucleus was observed using a Nikon fluorescence microscope and photographed using Ilford 400 ASA film.

2.7 CYANOGEN BROMIDE ACTIVATION OF SEPHAROSE-4B:

Sepharose 4B was activated following the procedure of March et al., (1974) with some modifications. Sepharose 4B was washed with double distilled water (DDW), followed by 1M sodium bicarbonate, pH 10.9. The volume of Sepharose 4B was measured and two volumes of 1M sodium bicarbonate, pH 10.9 was added. The slurry was stirred slowly on ice to keep the temperature at 4°C. The following operations were all carried out in a fume hood. The rate of stirring was increased and 0.05 volumes of cyanogen bromide solution (2 mg CNBr/ml acetonitrile) was added. The slurry was stirred vigorously for 10 minutes at 4°C. The slurry was poured into a sintered glass funnel and washed with 10 volumes each of 0.1 M sodium bicarbonate, pH 9.5; double distilled water and 0.4 M sodium bicarbonate, pH 9.5. After the last wash, the slurry was filtered to a moist cake. The activated Sepharose 4B was transferred to a bottle containing one volume of the protein solution. The protein to be coupled to Sepharose 4B was dissolved in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M sodium chloride. Coupling was done at 4°C for 20 hours with gentle stirring on a rotary shaker. The protein solution was removed, Sepharose was washed with 1 M glycine and stirred with 1 M glycine for an additional four hours to mask the unreacted groups. The protein-Sepharose was washed with 20 volumes each of 0.1 M sodium acetate, pH 4.0 and 0.1 M sodium bicarbonate, pH 8.3; both the buffers contained 0.5 M sodium chloride. The matrix was subsequently washed with the TEMN buffer and stored at 4°C in TEMN buffer containing 0.02% sodium azide.
2.8 PREPARATION OF ss-DNA-SEPHAROSE.

DNA was isolated from goat liver following the procedure of Marmur (1961). DNA (1g) was dissolved in 25 ml of 0.5 N sodium hydroxide and the solution was left in a boiling water bath for 15 minutes. The DNA solution was cooled on ice and 25 ml of 0.5 N HCl was added, followed by 50 ml of 0.4 M sodium bicarbonate pH 9.5. This was mixed with 100 ml CNBr activated Sepharose 4B and the coupling was carried out as described above.

2.9 PREPARATION OF ACTIN-SEPHAROSE.

Actin was purified from the goat skeletal muscle following the procedure of Pardee and Spudich (1982). Acetone powder of the skeletal muscle was made as follows. 250 g muscle was washed with double distilled water and minced finely. The mince was washed extensively with distilled water and extracted for 10 minutes in 500 ml ice cold 150 mM potassium phosphate buffer pH 6.5 containing 100 mM potassium chloride. This was filtered under vacuum. The residue was extracted by stirring successively in 1) 1000 ml of 50 mM sodium bicarbonate for 10 min; 2) 500 ml of 1 mM EDTA pH 7.0 for 10 min; 3) twice with 500 ml of double distilled water for 5 min and 4) five times each with 250 ml of acetone (at room temperature) for 10 mins. Each step was followed by filtration under vacuum. The final residue was lyophilized in order to remove traces of acetone. The acetone powder was obtained and stored at -70°C until further use.

Ten grams of acetone powder was extracted with 250 ml TMC buffer containing 0.2 mM ATP for 30 mins. The mixture was centrifuged at 15,000 X g for 20 mins. The supernatant was aspirated very carefully, leaving the turbid layer in the centrifuge tube. To the supernatant potassium chloride, magnesium chloride
and ATP were added to final concentrations of 50 mM, 2mM and 1 mM respectively. The supernatant was stirred with a glass rod and left undisturbed for 2 hours. The KCl concentration of the supernatant was increased to 0.6 M and the stirring was continued for an additional one and a half hours. The polymerized actin was pelleted by centrifugation at 80,000 X g for 3 hours. The pellet was homogenized in TMC buffer containing 0.2 mM ATP and dialyzed for 3 days against the same buffer in order to depolymerise actin. The dialysate was centrifuged at 80,000 X g for 3 hours. The supernatant was collected and chromatographed over a column of Whatman DE-52, pre-equilibrated with imidazole buffer containing 100 mM KCl. The column was washed with the same buffer. Elution was carried out with 300 mM KCl in imidazole buffer. The eluate was dialyzed overnight against 2 liters of TMC buffer containing 50 mM KCl, 2mM MgCl₂ and 1 mM ATP to effect actin polymerization. The dialysate was centrifuged in order to pellet actin at 80,000 X g for 3 hours. The pellet was resuspended in TMC buffer containing 0.2 mM ATP and was dialyzed against the same buffer for two days. The dialysate was centrifuged at 80,000 X g for 3 hours. The supernatant which contained pure actin was lyophilized. Actin was dissolved in 0.2 M sodium bicarbonate, pH 8.3 containing 0.5 M NaCl and coupled to CNBr activated Sepharose 4B as described above.

2.10 PREPARATION OF ESTROGEN RECEPTOR-SEPHAROSE.

The activated ER was purified from goat uterine cytosol following the procedure of Zafar and Thampan (1993). All the chromatographic procedures were performed using batch adsorptions and elutions. Cytosol was prepared in TEMN buffer. The cytosol was mixed with a Whatman DE-52 suspension equilibrated with TEMN buffer. The matrix was washed with TEMN buffer and eluted with
TEM buffer containing 0.2 M NaCl. The eluate was mixed with phosphocellulose. The phosphocellulose was washed with TEM buffer containing 0.2 M NaCl and eluted with TEM buffer containing 0.3 M NaCl. The NaCl concentration in the phosphocellulose eluate was diluted to 0.1 M using TEM buffer following which the eluate was mixed with ss-DNA-Sepharose equilibrated with TEM buffer. DNA Sepharose was washed with TEM buffer and was eluted with TEM buffer containing 10 mM ATP. The ATP eluate contained the pure ER. The free ATP was dialyzed out against TEM buffer and the dialysate was lyophilized. ER obtained from several independent preparations was pooled and about 10 mg of the ER was dissolved in 10 ml of 0.2 M NaHCO₃ pH 9.5 containing 0.5 M NaCl and coupled to CNBr activated Sepharose 4B as described before.

2.11 PREPARATION OF ER-NLS-SEPHAROSE.

The ER-NLS peptide, which includes pNLS1, pNLS2 and pNLS3 along with the intervening sequences (amino acids from 256-303; RKDRRGGRMLKHKRQRDDGEGRGEVGSAAGDMRAANLWPSPLMIKRS KK) was synthesised on hydromethyl tetraethylene glycol diacrylate-crosslinked polystyrene support using standard Fmoc.solid phase peptide synthesis protocol (Renil et al., 1994). The coupling reactions were carried out using dicyclohexyl carodimide/N-Hydroxybenzo triazole coupling procedure using 3 fold excess of Fmoc protected amino acid derivatives. The stepwise deprotection were carried out using 20% piperidine in NN'Dimethyl foramide. Finally, after the synthesis the peptide was cleaved from the support using trifluoro acetic acid: thioanisol: m-cresol (10:1:1) mixture at 40°C for 6 hours. The peptide was purified by reprecipitating with diethylether and directly used for coupling reaction with CNBr activated Sepharose 4B.
2.12 PREPARATION OF ER-NLS (CONTROL)-SEPHAROSE.

The ER-NLS (control) peptide, which contains the same sequence as for the ER-NLS peptide but all the three NLSs were substituted with amino acid alanine residues. (Amino acid sequence \textit{AAAAAGGRM\overline{AAAAAA}\overline{AGEGRGEGVGSAG}} \textit{DMRAANLWPSPLML\overline{AAAAA}}) was synthesized according to the procedure described by Renil et.al (1994).

2.13 PREPARATION OF ER-HBD-SEPHAROSE.

The ER-HBD peptide (amino acids 302-320, "\textit{KKNLASLTADQMVSALL}") was synthesized according to the procedure described by Renil et.al (1994). 10 mg of the synthetic peptide was directly coupled to CNBr activated Sepharose 4B.

2.14 PREPARATION OF p55-SEPHAROSE.

p55 was purified following the procedure of Nirmala and Thampan (1995a) with slight modifications. Cytosol was prepared in TEMN buffer. The cytosol was chromatographed over a 10 ml column of actin-Sepharose. The column was washed extensively with TEMN buffer and the p55 bound to the column was eluted with TEM buffer containing 0.8 M NaCl. There was no indication for the presence of the 48 kDa protein (which was often present in the procedure reported earlier) in my preparation. The NaCl eluate contained highly purified p55. The salt was dialyzed out following which the dialysate was lyophilized. About 5 mg of p55 was coupled to 7 ml of CNBr activated Sepharose 4B as described above.
2.15 PREPARATION OF p12-SEPHAROSE.

The purification of p12 was described in section 4.2. About 10 mg of p12 was coupled to 7 ml of CNBr activated Sepharose 4B.

2.16 PREPARATION OF WHEAT GERM AGGLUTININ (WGA)-SEPHAROSE.

WGA was purified according to the procedure described by Nagata and Burger (1974). Wheat germ was finely powdered and stirred in 10 volumes of 0.05 N HCl for one hour at room temperature. The suspension was centrifuged at 4000 X g for 10 minutes. To the supernatant solid ammonium sulphate was added to 35% saturation and the mixture was stirred for one hour at 4°C. The precipitated proteins were collected by centrifugation at 9000 X g for 15 minutes. The precipitate was dissolved in 0.05 N HCl and butanol was added dropwise with constant stirring at room temperature to a final concentration of 20% (v/v). The stirring was continued for another one hour and the preparation was centrifuged at 5000 X g for 30 minutes at 4°C. The aqueous phase was collected and was subjected to the butanol extraction twice again. The aqueous phase was dialyzed overnight against two changes of 0.05 N HCl. Ammonium sulfate was added at 35% saturation to this extract as described above. The precipitate was resuspended in a small volume of 0.05 N HCl and was dialyzed overnight against 0.01 M Tris-HCl, pH 8.5. The dialysate was centrifuged at 10,000 X g for 15 min and was chromatographed on a DE-52 column equilibrated with 0.01 M Tris-HCl, pH 8.5. Elution of WGA from the column was achieved using the same buffer. WGA appeared in the flow through fraction of the DE-52 column. 10 ml fractions were collected and the absorbance was measured at 280 nm. The peak absorbance fractions were pooled, dialyzed overnight against distilled water and lyophilized.
The lyophilized material was dissolved in minimum volume of 0.05 N HCl, neutralized carefully with NaOH and finally centrifuged at 5,000 X g for 10 min. Crystals appeared after 1or 2 days at 4°C. The crystals were harvested and rinsed with distilled water by low speed centrifugation and were recrystallized as above. WGA thus obtained was tested for homogeneity by SDS-PAGE and about 10 mg of the purified protein was coupled to CNBr activated Sepharose 4B.

2.17 PREPARATION OF CONCANAVALIN-A(CON-A) SEPHAROSE.

10 mg of commercially available Con A was coupled to CNBr activated Sepharose 4B following the procedure of March et al. (1974).

2.18 FITC- LABELLING OF ER, p55 and p12.

Goat uterine estrogen receptor was purified following the procedure described by Zafar and Thampan (1993). The ER was labeled with FITC following the procedure of Chard (1987). One mg of ER was dissolved in 250 μl of 0.15 M Na₂PO₄·2H₂O pH 9.5. 100 μl of the FITC solution (1 mg/ml) in the same buffer was added to the protein solution. The pH of the mixture was adjusted to 9.5 with 1M Tris and the mixture was incubated overnight at 4°C. The unbound FITC was removed following chromatography of the mixture on a column of Sephadex G-25.

Goat uterine p55 was purified following the procedure of Nirmala and Thampan (1995a). The p55 was labeled with FITC following the procedure of Chard (1987) as described above.

P12 was purified from goat uterine nuclei (described in section 4.2) and labeled with FITC as described above.
2.19 IMMUNIZATION PROTOCOL.

The following immunization protocol was followed. The protein under study was dissolved in 10 mM Tris buffer pH 7.6 and was mixed with Freund’s complete adjuvant (in the ratio 1:1). The mixing was done thoroughly to make a thick colloidal suspension. Two ml of the suspension was injected into five different subcutaneous locations and also into the foot pad of a New Zealand white rabbit. The second injection (first booster) was given 15 days after the first. Protein for the booster injections was prepared by mixing the protein solution with Freund’s incomplete adjuvant in the ratio 1:1. The second and the third boosters were given at 15 days intervals. The rabbits were bled a week after the second booster. The serum was collected and tested for the presence of antibodies by western blotting analysis. The rabbits were given another booster injection and bled from the ear vein a week later. The serum was isolated, aliquoted and stored at -20°C.

Antibodies were raised against goat uterine estrogen receptor, p55 and p12.

2.20 ISOLATION OF IgG FROM THE ANTISERUM.

The IgG was purified following chromatography of the serum on a column of protein A-Sepharose as described by Sambrook et al. (1989). The antiserum was mixed with 0.1 volume of 1 M Tris-HCl pH 8.0 and was chromatographed on a column of protein A-Sepharose (3 ml), equilibrated with 0.1 M Tris-HCl pH 8.0. The column was washed with ten volumes of 0.1 M Tris-HCl pH 8.0 followed by ten volumes of 10 mM Tris-HCl, pH 8.0. Elution was achieved using 100 mM glycine-HCl, pH 3.0. The fractions (500μl) were collected in tubes containing 50μl of 1 M Tris-HCl pH 8.0. The absorbance of the fractions at 280 nm was
monitored. The IgG was eluted as a single peak of absorbance at 280 nm. The fractions containing the IgG were pooled and dialyzed extensively against 10 mM Tris-HCl pH 8.0 in order to remove glycine.

2.21 PREPARATION OF PROTEINS FOR SDS-PAGE.

To the protein solution 100% TCA was added to give a final TCA concentration of 30%. The solution was mixed thoroughly and left on ice for a minimum of two hours. The samples were transferred to clean Eppendorf tubes and were centrifuged at 12,000 X g for 20 min. The supernatant was decanted and the tubes were inverted over a blotting paper to drain off traces of TCA. The precipitate was then washed twice with distilled ethanol. The final precipitate was dried in air to remove alcohol and was dissolved in 20μl of SDS sample buffer. The samples were denatured following heating in a boiling water bath for 3 mins.

2.22 SDS-PAGE.

SDS-PAGE was performed as described by Laemmli (1970). Proteins were resolved either on standard gels (15cm X 13 cm X 0.1 cm) or mini gels (7.5cm X 8cm X 0.1cm) for rapid analysis.

Solutions used:

1) Acrylamide solution: 30 g acrylamide and 800 mg methylene bis-acrylamide were dissolved in double distilled water and the volume was adjusted to 100 ml. The Acrylamide solution was filtered through Whatman No.1 filter paper and stored in amber colored bottles at 4°C.

2) Lower Tris (4X)(Separating gel buffer)(1.5 M Tris-HCl pH 8.8 with 0.4% SDS); 18.17 g Tris was dissolved in double distilled water. To this 4 ml 10% SDS was added and the pH of the solution was adjusted to pH 8.8 with 12 N HCl. The
volume was then made up to 100 ml. The lower Tris was filtered through Whatman No.1 filter paper and was stored in glass bottles at 4°C.

3) **Upper Tris (4x) (Stacking gel buffer)** (0.5 M Tris-HCl pH 6.8 with 0.4% SDS): 6.06 g Tris was dissolved in double distilled water. To this 4 ml 10% SDS was added following which the pH of the mixture was adjusted to 6.8 with 12 N HCl. The final volume was then made up to 100 ml. The upper Tris was filtered and stored at 4°C as indicated above.

4) **Reservoir buffer (8x)**: 24 g Tris and 115.2 g glycine were dissolved in double distilled water and the volume was made up to a liter. The buffer was stored at 4°C and diluted just before use.

5) **Reservoir buffer (X)**: 125 ml of 8X reservoir buffer and 10 ml 10% SDS was diluted to 1000 ml with double distilled water.

6) **10% SDS**: 10 g SDS was dissolved in double distilled water and the volume was made up to 100 ml and stored at room temperature.

7) **10% APS**: 1 g APS (ammonium persulphate) was dissolved in 10 ml double distilled water. This solution was always prepared fresh before use.

8) **Sample buffer**: 1.0 ml glycerol, 5.0 ml β-mercaptoethanol, 3.0 ml 10% SDS and 1.25 ml upper Tris (4X) were mixed and the volume of the mixture was made up to 10 ml using double distilled water. 10 μg bromophenol blue was added to the sample buffer. The sample buffer was stored at -20°C as 1.0 ml aliquots.

**Electrophoresis**: Discontinuous standard slab gels or mini slab gels were polymerized just before use. Gels of 1 mm thickness were routinely used as 1 mm gels since they were easy to handle and were of the optimal thickness for silver staining. 5 to 15% gels were prepared as given in the table shown in the next page (the volumes of the solutions are given in milli litres).
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Standard gels were run at a constant current of 30 mA and the samples in mini gels at a constant voltage of 60 V through the stacking gels and 100 V through the separating gels. When the tracking dye reached the end of the gels, the run was terminated and the gels were transferred to the gel boxes containing the fixer. Standard proteins of known molecular mass were loaded into one of the wells to enable the calculation of the molecular mass of the unknown proteins.

### 2.23 SILVER STAINING OF SDS GELS.

The gels were stained with silver nitrate following the procedure of Blum et al. (1987). The solutions required for this procedure were all prepared fresh just before use. All the solutions were made in double distilled water.
1) **Fixer:** 50% methanol, 12% glacial acetic acid and 100 µl 37% formaldehyde/200 ml.

2) **50% ethyl alcohol.**

3) **Thiosulphate solution:** 40 mg sodium thiosulphate was dissolved in 200 ml double distilled water.

4) **Silver nitrate solution:** 400 mg silver nitrate was dissolved in 200 ml double distilled water. 150 µl of 37% formaldehyde was added to this solution immediately before use.

5) **Developer:** 12 gm of anhydrous sodium carbonate and 100 µl of 37% formaldehyde was dissolved in 200 ml double distilled water.

6) **12% glacial acetic acid.**

The gels were transferred to a staining tray containing 200 ml of the fixer and were left on a shaker for one hour. The gels were washed with 50% ethanol for 30 minutes. The ethanol was removed and the gels were treated with the thiosulphate solution for one minute. The treated gels were washed thrice with distilled water for about twenty seconds each in order to remove thiosulphate. The gels were then incubated with the silver nitrate solution for twenty minutes with continuous shaking. The silver nitrate solution was then drained off and the gels were washed thoroughly with double distilled water as mentioned above. The gels were then developed with the Na₂CO₃ solution (developer) till protein bands were seen, washed with double distilled water and left in 12% glacial acetic acid to stop staining.
2.24 WESTERN BLOTTING.

Western blotting analysis was performed according to the procedure developed by Towbin et al. (1979) and Yonezawa et al. (1988). The proteins were separated on mini SDS-PAGE gels. After electrophoresis the gels were immersed in cold Towbin buffer (25 mM Tris pH 8.3, 192 mM glycine and 20% methanol) for half an hour. The dimensions of the gel were taken and the nitrocellulose membrane of the same size was cut and equilibrated in Towbin buffer. Six Whatman No.3 sheets of the same size as of the gel were taken and equilibrated along with the fiber pads in Towbin buffer. A sandwich was made as follows: the grey panel, fiber pad, 3 layers of Whatman paper, gel, nitrocellulose membrane, 3 layers of Whatman paper, fiber pad and the white panel. Care was taken to remove any air bubbles trapped between the gel and the nitrocellulose membrane. The sandwich was clamped tightly and immersed in the electrophoresis tank filled with ice cold Towbin buffer, with the grey panel facing the cathode. The transfer was performed in a Trans Blot Electrophoretic cell (BIO-RAD 170-3910). The transfer was done at 70V (0.25A limit) for three hours. While the transfer was going on, the buffer in the tank was stirred to prevent local heating. After the transfer, the blots were air dried and washed with TNN buffer for 10 minutes. The blots were blocked with 3% BSA in TNN buffer for one hour, at room temperature. The blots were incubated overnight with the primary antibody in 3% BSA in TNN buffer. The blots were washed with TNN buffer and were re-incubated with anti-rabbit IgG (anti-mouse IgG in case of monoclonal primary antibody) coupled with horse radish peroxidase (HRPO) or alkaline phosphatase (AP) in 3% BSA in TNN buffer for two hours at room temperature. The unbound antibody was washed with TN buffer (TNN buffer without NP40) and stained with 4-chloro, 1-naphthol as substrate (10% methanol, 0.25% H₂O₂ and 0.3 mg/ml 4-chloro, 1-naphthol in TN
buffer) in the case of horse radish peroxidase-IgG, or, with 5-bromo 4-chloro 3-indolyl phosphate (BCIP) nitroblue tetrazolium (NBT) (0.85% NBT in dimethyl formamide and 5% BCIP in 50 mM Tris-HCl, pH 9.0 containing 150 mM NaCl and 100 mM MgCl₂ in the case of alkaline phosphatase-IgG.

2.25 GEL FILTRATION ANALYSIS.

A Sephadex G-100 column (75 x 1.5 cm) was used to perform gel filtration analysis in order to determine the Stokes radius (Rₛ) of the purified protein following the procedure of Siegel and Monty (1966). The Sephadex G-100 column was calibrated with the gel filtration markers (blue dextran and glycine) and proteins of known Stokes’s radii (bovine serum albumin (BSA), ovalbumin (OV) and cytochrome C (CYT C)). The column was equilibrated with TEM buffer containing 0.3 M NaCl. The marker proteins were dissolved (4 mg/ml) in the equilibration buffer and about 300 µl of this solution was applied carefully onto the column. Fractions collected were subjected to measurement of absorbance at 280 nm. The kd (distribution coefficient) of the protein was calculated and a standard graph of kd versus Rₛ of the marker proteins was made.

\[
k_d = \frac{V_e - V_o}{V_t - V_o}
\]

where,

\[V_o = \text{Volume of buffer used to elute blue dextran},\]
\[V_t = \text{Volume of buffer used to elute glycine},\]
\[V_e = \text{Volume of buffer used to elute a given protein } V_{e_{BSA}}, V_{e_{OV}}, V_{e_{CYT-C}} \text{ etc.}\]

The kd of the unknown protein was also monitored similarly and the Stokes radius of the protein was calculated with the help of a standard graph.
2.26 ESTIMATION OF INORGANIC PHOSPHATE (Pi).

Estimation of Inorganic Phosphate (Pi) was performed following the procedure of Fiske and Subbarow (1925) with some modifications.

Reagents used:
1) 20 μg/ml potassium phosphate solution (for the standard) in 0.05 N hydrochloric acid.
2) 0.05 N hydrochloric acid.
3) Acid molybdate: 1.25% ammonium molybdate in 2.5 N sulphuric acid.
4) Fiske-Subbarow reagent: 7.312 g sodium metabisulphate, 400 mg 1-amino-2-naphthol-4-sulphoic acid (ANSA) and 250 mg sodium sulphite were ground well together in a porcelain crucible. This powder was stored in an amber-colored bottle. One g of this powder was dissolved in 25 ml double distilled water. The reagent was prepared immediately before use.

Standard phosphate solution was diluted with 0.05 N HCl to different concentrations (2 μg to 20 μg/ml). To the phosphate solution, 1 ml acid molybdate was added and mixed. This was followed by the addition of 250 μl of the Fiske-Subbarow reagent. The mixture was left at room temperature for 10 minutes. The blue color developed was measured at 660 nm against a blank containing 500 μl 0.05 N HCl that was treated similarly. An absorbance of 0.76 for 10 μg phosphate was taken as the standard value.

2.27 ATPase ASSAY:

The proteins p55 and p12 were incubated independently with 6 mM ATP in TMg buffer at 30°C for 30 mins with different protein concentrations. The volume
of the incubation mixture was 250 μl. The incubation was terminated with the addition of equal volume of ice cold 20% TCA. The tubes were left on ice for an hour for optimal precipitation of the proteins. The supernatants were isolated following centrifugation in the cold at 10,000 X g for 20 minutes. The Pi present in the supernatants was estimated as described above. The concentration of the Pi released was calculated from the standard graph and the value was converted to its molar concentration (20 μg/ml is 0.65 mmol/l). The ATPase activity was expressed as μmoles Pi released/hour.

2.28. ESTIMATION OF PROTEINS:

Proteins were estimated following the procedure of Bradford (1976). 100 μl of the test sample was mixed with 1 ml of Bradford's reagent (10 mg of Coomassie Brilliant Blue G-250 was dissolved in 5 ml of ethanol. To this 10 ml of 85% (w/v) phosphoric acid was added. The resulting solution was made upto 100 ml with DDW and was filtered through Whatman No.1 filter paper). After 5 minutes the color developed was measured at 595 nm. BSA was used as the protein standard.