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SUBCELLULAR DISTRIBUTION AND PURIFICATION OF HEAT SHOCK PROTEIN 90 IN SHEEP LIVER TISSUE

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ABSTRACT

Heat shock proteins are a group of highly conserved proteins that are constitutively expressed in most cells under normal physiological conditions.HSP90 has been identified in the cytosol, nucleus and, endoplasmic reticulum, and is reported to exist in many cell types. To obtain large amount of HSP 90 and examine the properties of the purified protein, tissues may be better as materials for purification than cultured cells. The 90KDa heat shock protein (HSP90) has been purified from mammalian tissues(sheep liver) with a good yield by a method involving DEAE-cellulose chromatography, and its subcellular localization in different organelles reveals that HSP90 is a highly conserved protein during evolution. Our present study examines the constitutive expression of HSP90 in various subcellular fractions of liver tissue by immunobloting and its purification by conventional chromatographic techniques.

KEYWORDS: Hsp, DEAE, Sheep liver, chromatography.

INTRODUCTION

Heat shock proteins are highly conserved proteins which are induced in cells upon exposure to elevated tempreture or other forms of cellular stress (subjeck and shyy 1986, Welch 1992, parsell and Lindquist 1993). In addition, most of HSPs are also present in the unstressesd cell, where they are thought to play a vital role in normal cellular function (Lindquist and craig 1988). One of the main role that heat shock proteins play is that of a

molecular chaperone. Specifically, heat shock proteins (HSPs) have been shown to function in protein maturation events such as protein folding, unfolding and translocation across membrane (ellis and vandervies 1991, gething and sambrook). HSP90 is an abundant cytosolic protein which is found in association with variety of other intracellular proteins including calmoduln(minami et al 1988,) Actin(nishida et al 1986), Tubulin(sanchez et al 1988). several kinases (oppermann et al 1981, rose et al 1987), and steroid receptor (kang et al 1994).Among other functions,HSP90 stabilizes target proteins in an inactive or unassembled state.

MATERIALS AND METHODS

Isolation of protein homogenates

Liver from healthy sheep was obtained from local abattoir immediately after slaughter and chilled crushed. All steps were carried out at 0-4c and homogenized in 0.32M sucrose. Protein concentrations were determined using bradford method.

One-dimensional gel electrophoresis and western blot procedure

Protein samples were solubilized by boiling for 5min with an equal volume of dissociation buffer (8M urea, 2% SDS,2% mercaptoethanol, 20% glycerol).

Polyacrylamide gel electrophoresis was carried out in the presence of SDS on 10% (for HSP 90) gels with 5% stacking gels using the discontinuous buffer system of laemmli (1970). The proteins were transferred onto PVDF membrane for 16-18h in a solution of 50mM boric acid, 4mm mercaptoethanol and 2mM EDTA, at 400mA.For western blotting, the blots were washed 4x5min in TBST buffer (10mM Tris, 0.25mM NaCl, 0.5%t tween 20, ph 7.5) blocked for 1h at room tempreture in 5% carnation milk powder in TBST buffer, then incubated overnight in primary antibody. The antibodies were used polyclonal, anti hsp 90. Following incubation with primary antibody blots were washed 4x10min in 1% BSA (sigma) in TBST, incubated for 2h at room tempreture with secondary antibody, with antirabbit IgG diluted 1;10,000 (polyclonal antibody) and then washed 6x5 min in TBST. Immunoreactive bands werevisualized by western blotting detection reagents.

Preparation of nuclear and mitochondrial fractions

Nuclear protein extract were prepared according to the method of zhu et al (2001). To the nuclear pellets, ice cold high salt buffer containing (20mM Hepes, 25% glycerol,

0.42M NaCl, 0.2mMEDTA, 1.5Mgcl2, 0.5mMPMSF). was added and mixed nuclei were incubated for 15 minutes and centrifuged at maximum speed for 1 minute. Supernatant were saved as nuclear protein extract. Mitochondrial fractions were obtained when the post nuclear supernatant i;e, (the supernatant of the first centrifugation), is centrifuged at 5000g for 10 minute.

Protein estimation

Protein concentrations at each step of the purification was determined by Bradford method using bovine serum albumin as standard

RESULTS AND DISCUSSIONS

Allsteps were carried out at 0-4c. An extract of cytosolic proteins were prepared by homogenizing 50g of tissue in 150ml of 0.03 carbonate bufferPh 7.1 containing 5mM PMSF and 5mg/ml aprotinin. The homogenate was cleared by ultracentrifugation and subjected to ammonium sulfate precipitation at 50% after which the supernatant was brought to 70% saturation. The precipitate was dissolved and dialyzed extensively against 0.2M NaCl. The solubilized proteins were then subjected to anion- exchange chromatography using a gradient of NaCl from 0.2 to 0.6M in phosphate buffer ph 7.4. The fractions with the highest content of protein that migrated at 90KDaupon SDS page were pooled. Fractions containing HSP90 at apparent homogeneity were pooled dialyzed against PBS, aliquoted and kept at -70c, until use. Purified HSP90 stained as a single protein band of 85-90 KDa.

Figure 1: Western Blot showing the presence of HSP 90. From left to right: Fig1:Lane 1: M.Wt markers, L-2: HSP 90 in whole cell Homogenate, L-3 purified fraction.(volume loaded in each lane 30µl).

Supernatant was applied to anion exchange column(4cmx2mm) equilibrated with buffer solution containing (1mM EGTA , 0.5mM DTT and 10 mM Tris/HCl, Ph 7.5). After washing the column with the same solution,until the absorbance of the eluate decreased to less than 0.025 at 280nm. The adsorbed proteins were eluted with a linear gradient of gradient of 0-500mM Nacl in same solution at a flow rate of 25ml/hour. Fractions containing hsp 90 were pooled and stored at -70c.

Identification of 90 KDa HSP in various subcellular fractions of liver tissue

Different cellular fractions were prepared as described in the materials and methods section. A protein was identified of apparent molecular mass 90KDa, eluted at 80mM NaCl and merited further investigation in other subcellular organelles of liver tissue by western blotting. Equal amounts of protein (20µg/lane) were saperated by 10%(w/v) SDs Page and then resolved proteins were transferred to PVDF membrane for 16-18h in a solution of 50mM boric Acid, 4mM Beta mercaptoethanol and 2mM EDTA. The non-specific binding sites on membrane was blocked by using 5% carnation milk powder in TBST buffer and then incubated with anti hsp90 polyclonalantibody (2µl) and probed with secondary antibody coupled to alkaline phosphatase. The reactivities were revealed using NBT/BCIP chromogenic substrate.

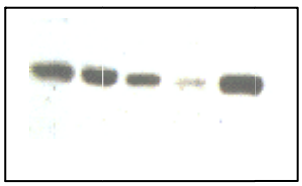

Figure 2: Western Blot showing distribution of HSP 90 in various subcellular factions of liver tissue. From left to right: L-1 whole cell homogenate, L 2: cytosolic fraction, L-3: Nuclear fraction, L-4 mitochondrial faction, L-5 Purified fraction. Volume loaded in each lane(20µl). Distributional analysis of 90KDaheat shock protein in various subcellular fractions of liver tissue was undertaken by western blot analysis and protein appeared to be ubiquitously distributed in various fractions viz: cytosolic, mitochondrial, nuclear fractions substantiating its wide distribution consistent with its basic role as multifunctional molecular chaperone.

CONCLUSION

This study has demonstrated the isolation of hsp 90 in sheep liver tissue and it remains only one step away to significantly improve upon the existing information about hsp fraction in liver tissue.

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