

An efficient procedure for purification of recombinant human β heat shock protein 90

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ABSTRACT

Background and the purpose of the study: Heat Shock Protein 90 (Hsp90) is typically the most abundant chaperone in the eukaryotic cell cytoplasm, and its expression is essential for loading immunogenic peptides onto major histocompatibility complex molecules for presentation to T-cells. Therefore, it may act as a good candidate as an adjuvant molecule in vaccine technology.

Methods: Initially the human Hsp90 β gene was cloned into the heat inducible expression vector pGP1-2 and then the recombinant protein was isolated by ion exchange chromatography. After intradermal injection of confirmed purified band of protein to rabbits and isolation of the serum IgG antibody, for its affinity purification, the rabbit's purified Hsp90 specific IgG was coupled to the cyanogen bromide-activated Sepharose 4B.

Results: The recovery of the purified protein of interest by affinity chromatography was 50%.

Conclusion: This research enabled purification of human heat shock protein by a laboratory prepared column chromatography.

Keyword: Heat shock protein 90(Hsp90), CNBr activated Sepharose 4B, Rabbit IgG, pGP1-2

INTRODUCTION

Hsp90 (heat shock protein 90) is a molecular chaperone that modulates the stability and/or transport of a diverse set of critical cellular regulatory including metabolism, organization, and signaling proteins. Binding to Hsp90 is required for normal function of many proteins (1). In addition purified heat shock proteins (Hsps) can be applied in several aspects of biology, medicine and protein biotechnology. In mammalian cells, there are two genes encoding cytosolic Hsp90 homologues with the human Hsp90 α which show 85% sequence identity to Hsp90 β (2,3). The protein has been implicated in loading immunogenic peptides onto major histocompatibility complex molecules for presentation to T cells(4,5). They are immunogenic and can be used for anticancer vaccines(6).

Immunoaffinity purification is a highly specific and reversible technique(7) which was used in this investigation to produce the recombinant Hsp90 without using any chemicals as a convenient adjuvant and chaperone in human vaccines.

MATERIAL AND METHODS

Cell line and Plasmids

The MDA-MB468 cell line was obtained from the

Pasteur Institute of Iran. *Escherichia coli* strains *NovaBlue* and *DH5 α* were purchased from Invitrogen, USA. *pTZ57R* (Fermentas, Germany) and *pGP1-2* (donated by Dr. Bagher Yhakhchali NIGEB, Tehran, Iran) were plasmids which were used in this study. Human Hsp90 polyclonal antibody was purchased from Abcam, UK.

Cell culture and stimulation

The MDA-MB468 cell line was maintained in RPMI (Roswell Park Memorial Institute, Sigma, Germany) containing 2mM L-glutamine (Sigma, Germany), 100 U/ml penicillin (Sigma, Germany), and 100 μ g/ml streptomycin (Sigma, Germany) supplemented with 10% fetal bovine serum(Gibco,UK)(8). The plateau level for these cultures was about one million viable cells/ml. The sufficient flasks were induced by 50 ng of phorbol myristate acetate (PMA)(Sigma, Germany) without any physical manipulation(9).

RT-PCR and Cloning into the pGP1-2 vector

The MDA-MB468 cell line was analyzed for Hsp90 expression. The total RNA was extracted by RNeasy mini kit (Qiagen, USA ,74104) and the generation of cDNA and PCR was carried out by C.therm Polymerase One-Step RT-PCR System (Roche,

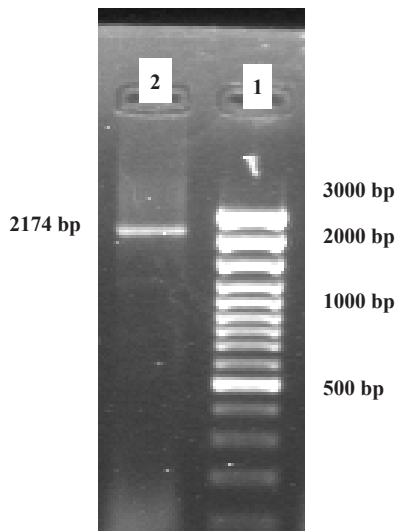


Figure 1: PCR product of Hsp90 β on 1% agarose gel
Lane 1: 100bp DNA Ladder (Fermentas, Germany)
Lane 2: 2174 bp Hsp90 PCR product

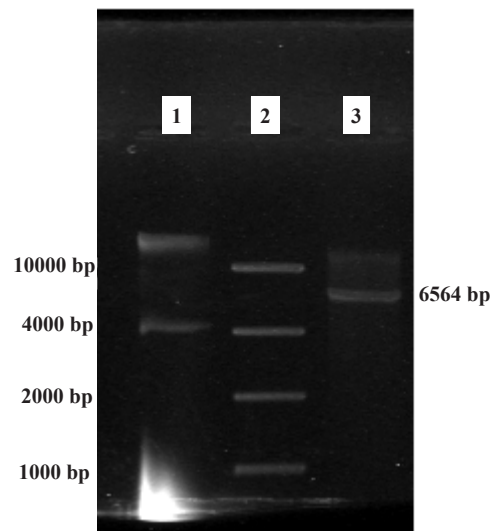


Figure 2: pGp1-2 subcloning confirmation
Lane 1: Undigested cloned plasmid
Lane 2: Gene Ruler 1kb DNA Ladder (Fermentas, Germany)
Lane 3: Digested plasmid by DraIII (linear form)

Germany, 12016338001). Specific primers were designed by GenRunner software: H90F, 5' TC CGG ACC TGA GGA AGT GCA CCA 3' and H90R, 5' GGT NAC CCT AAT CGA CTT CTT CCA TGC 3' (10). The Hsp90 gene PCR product was cloned into the *pTZ57R* T-vector (Fermentas, Lithuania) as described previously (11). The recombinant plasmid was constructed by using the *Kpn*2I and *Bst*EII restriction enzymes (Fermentas, Lithuania).

Expression, primary purification and analysis of the human Hsp90 β subunit

The *Nova Blue* strain of *E. coli* was transformed using above construct and protein expression was performed in LB (Lorian Bertani) medium containing 15 μ g/ml of kanamycine (Fermentas, Lithuania) (12,13). The heat induced culture of expressed *Novablue* strain of *E. coli* with *pGpHsp* (*pGPI-2* & human Hsp90 β subunit) was prepared and analyzed as described previously (14).

Polyclonal antibody production in Rabbits

The purified Hsp90 β (10 μ g) prepared from acrylamide as described previously (15) was used for immunization of rabbits subcutaneously. The serum antibody response was determined after secondary immunization by double diffusion test and also following booster injection (16).

Preparation of the Hsp90 β -specific IgG Sepharose 4B column

In this stage the common carotid artery of injected rabbits was used to collect large amount of blood

after anesthetizing by 100-150 mg/kg ketamine (Merck, Germany). The IgG antibody of rabbits sera was isolated by column chromatography using 3ml protein A resin (17). Rabbit's purified IgG was used as ligand to design the affinity chromatography column, as follow. For affinity purification of antibodies, immunoabsorbent columns were prepared with Hsp90 β coupled to cyanogen bromide-activated Sepharose 4B (GE Healthcare Bio-sciences, Sweden, 17-0430-01) (www6.gelifesciences.com). The above procedure allowed coupling of all specific IgG antibody (IgG pl 3-9) against HSP90 protein which were coupled to cyanogen bromide-activated Sepharose 4B.

Final Hsp90 β purification by Hsp-specific IgG Sepharose 4B column

The heat induced culture was harvested and the extracted protein was used for purification of the Hsp90 by the prepared affinity column as described above. The proceed lysate (10ml) was loaded onto the immunoabsorbent column at a flow rate of 0.5 ml/min. Bound proteins were eluted using a glycine-HCl (0.1M) and 2M NaCl at pH 5 (www6.gelifesciences.com) and confirmed by the double diffusion test and western blotting using a human Hsp90 polyclonal antibody.

RESULTS

The present study demonstrates isolation of the recombinant human Hsp90 β subunit by ion exchange chromatography after cloning and expression of the RT-PCR product (Fig1). Figure 1 shows the 2174 bp PCR product representing the human Hsp90 gene.

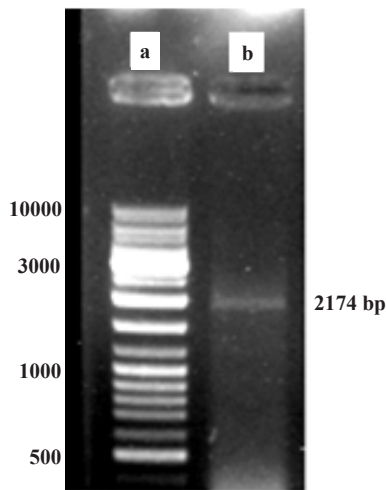


Figure 3: Confirmation of Hsp90 Cloning into the pGPI-2 by specific PCR
 Lane a: Gene Ruler 1kb DNA Ladder (Fermentas, Germany)
 Lane b: Direct PCR product of cloned plasmid

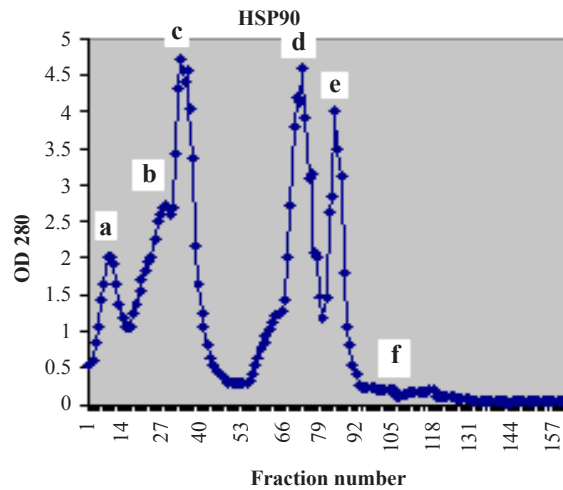


Figure 4: Showing Hsp90 β subunit purification chromatogram of fractions obtained from ion exchange chromatography of expressed construct
 a, b: Loading & washing stages
 c: Elution stage
 d, e, f: Washing stage

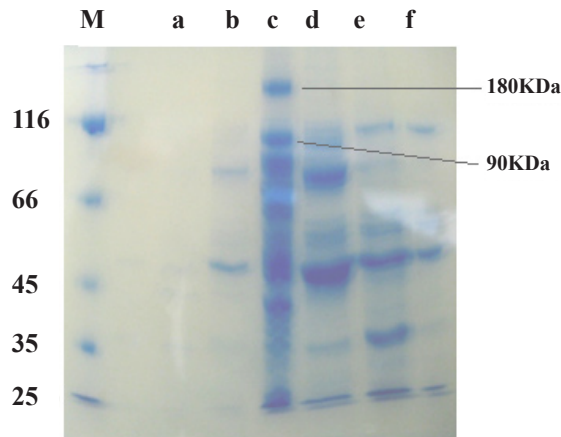


Figure 5: 10% SDS-PAGE of obtained fractions which are the peaks signed in fig.4. It was showed 90kDa protein band in lane c.

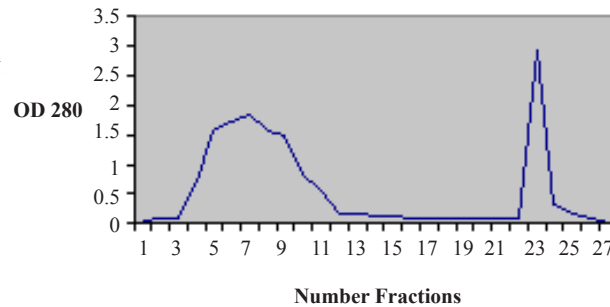


Figure 6: Chromatogram of IgG purification from serum by protein A affinity chromatography
 1- 3 –11: Loading
 2- 12- 22: Washing
 3- 23 – 25: Elution
 4- 26–30: Washing

Figure 2 shows confirmation of subcloning of Hsp90 β segment by *Dra*II restriction enzyme digestion. Also, this vector contained the kanamycine resistance gene and after transformation, bacteria could grow on medium supplemented with 30 μ g/ml kanamycine. The resulting clone was amplified using Hsp90 specific primers (Fig 3).
 The 160 fractions obtained from ion exchange chromatography of the expressed bacterial cells were determined by a biophotometer in 280 nm (Fig 4&5). According to our findings the chromatography parameters were regularized as: resolution 2, selectivity 2.5, efficiency 4 and capacity 30%.
 The Hsp90 protein required for preparation of a

specific affinity chromatographic procedure was obtained by gel extraction of 90kDa band of fraction C (in figure 4) and confirmed by western blotting using polyclonal antibody against Hsp90 β subunit (Fig 9).
 Figures 6 & 7 show the result of IgG isolation by different stages of the protein A column chromatography and its electrophoresis with non-reducing gel. While in the native IgG sample (lane c), a single predominant band is observed, the reduced IgG sample by DTT in lane b shows two bands related to light and heavy chains of antibody. In order to obtain pure Hsp90 by simple affinity chromatography with recovery 50%, the interested

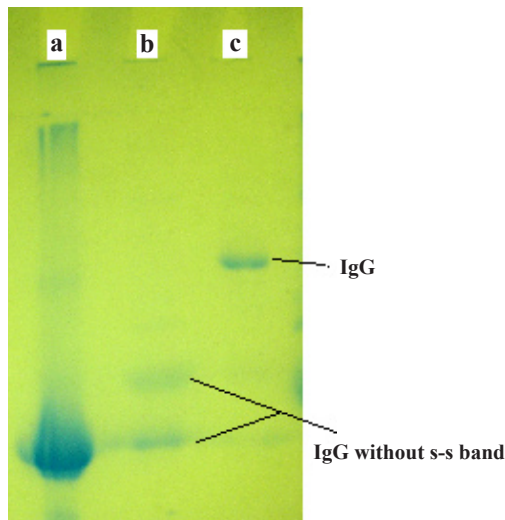


Figure 7: 10% SDS-PAGE of IgG after Protein A affinity chromatography
Lane a: Total serum proteins
Lane b: Boiling with SDS sample buffer
Lane c: Purified IgG

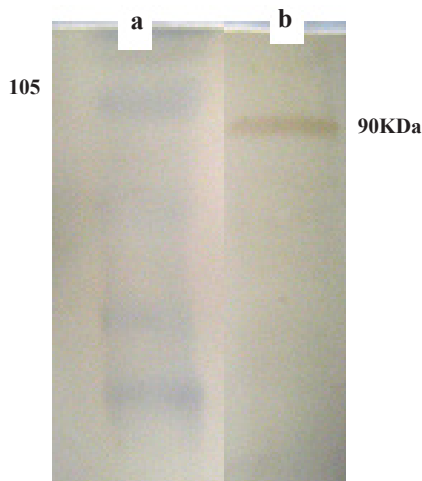


Figure 9: Western Blotting of recombinant protein purified by β Hsp90-specific sepharose 4B using polyclonal antibody

protein was identified by double diffusion and western blotting tests (Fig. 8, 9).

DISCUSSION

Hsps have a variety of important functions in immunity (5) and stimulate a wide range of innate and adaptive immune responses. The peptide binding function allows chaperon proteins such as Hsp90, Hsp70, and gp96 to acquire antigenic proteins within the cells when administered outside of the cell to induce priming of CD8⁺ T lymphocytes *in vivo* (18). The main aim of the present study was to establish

an efficient and easy-to-use method for purification of Hsp90 β . In immunoaffinity chromatography which can be considered as a subset of affinity chromatography, an immunoglobulin is used to bind analyte molecules. Because immunoglobulins show exquisite specificities towards antigens which are

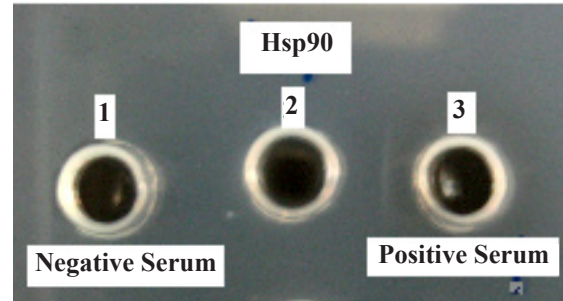


Figure 8: Immuno diffusion test using injected rabbit serum (+) and negative control (-)
1- Rabbit's serum without injection
2- Purified Hsp90 band by gel extraction
3- Immunized rabbit's serum

used in their production, immunoaffinity columns are ideal for purification of the targeted analytes (3). This study proposes that the specific IgG antibody against Hsp90 protein could be used for its purification by affinity chromatography; is highly selective for Hsp90 and has an appropriate affinity for this molecule. This strategy in which antibodies are immobilized in a single orientation generally involves immobilization of an intermediate protein such as protein A that specifically binds to the Fc region of an antibody and it can be coupled to a suitable commercially available matrix without affinity loss. In this study, the *pGp1-2* expression vector was initially used for production of Hsp90. A heat-induced transition of Hsp90 was then necessary to exert the chaperone activity (18). This treatment which caused self-oligomerization of Hsp90 under substrate-free conditions indicates a close relationship between self-oligomerization and the substrate-binding properties. Many *in vitro* studies on the chaperone function of Hsp90 have been performed around 25°C and are triggered by the addition of denatured proteins in renatured buffers containing Hsp90 without other chaperones and co-chaperones (19).

CONCLUSIONS

In this study natural and oligomeric forms of the Hsp90 β subunit were produced by the designed affinity chromatographic purification procedure without using any tag peptides or other materials such as IPTG leading to 50% recovery. In future chaperoning and adjuvant effects of Hsp90 in recombinant vaccines will be investigated.

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REFERENCES

1. Barginear MF, Van PC, Rosen N, Modi S, Hudis CA, Budman DR. The heat shock protein 90 chaperone complex: an evolving therapeutic target. *Curr Cancer Drug Tar*, 2008; 8:522-532.
2. Smith DF, Whiteseli L, Katsanis E. Molecular Chaperones: Biology and Prospects for Pharmacological Intervention. *Pharmacol Rev*, 1998; 4: 493-514.
3. Minami M, Nakamura M, Emori Y, Minami Y. Both the N- and C- terminal chaperone sites of Hsp90 participate in protein refolding. *Eur J Biochem*, 2001; 268: 2520-2524.
4. Przepiorka D, Srivastava PK. Heat Shock protein-peptide complexes as immunotherapy for human cancer. *Mol Med Today*, 1998; 4: 478-484.
5. Basu S & Srivastava P. Fever-like temperature induces maturation of dendritic cells through induction of Hsp90. *Int Immunol*, 2003; 15: 1053-1061.
6. Calderwood SK, Cicca DR. Heat shock proteins: stress proteins with janus-like properties in cancer. *Int J Hyperther*, 2008; 24: 31-39.
7. Asea A & Calderwood S. Heat Shock Proteins: Potent Mediators of Inflammation and Immunity. The Netherlands, Springer. 2007; 1: 61-100.
8. Pashov A, Kenderov A, Kyurkchiev S, Kehayov I, Hristova S, Lacroix S, Giltiy N, Varamballi S, Kazatchkine MD, Kaveri SV. Auto antibodies to heat shock protein 90 in the human natural antibody repertoire. *Int Immunol*, 2002; 14: 453-461.
9. Purcell AW, Todd A, Kinoshita G, Lynch TA, Keech CL, Gething MJ, Gordon TP. Association of stress proteins with autoantigens: a possible mechanism for triggering autoimmunity? *Clin Exp Immunol*, 2003; 132: 193-200.
10. Innis MA, Gelfand DH, Sninsky JJ. PCR Strategies. London, Academic Press. 1995; 58-65.
11. Kazemi B, Bandehpour M, Maghen L, Solgi G. Gene Cloning of 30 kDa *Toxoplasma gondii* Tachyzoites Surface Antigen (SAG1). *Iran J Parasitol*, 2007; 2: 1 - 8.
12. Sorensen H & Mortensen K. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *J Biotechnol*, 2005; 115:113-128.
13. Baneyx F. Recombinant protein expression in *Escherichia coli*. *Curr Opin Biotech*, 1999; 10:411-421.
14. Bandehpour M, Seyed N, Shadnoosh M, Pakzad P, Kazemi B. Using recombinant *Chlamydia* Major Outer Membrane Protein (MOMP) in ELISA diagnostic kit. *Iran J Biotech*, 2006; 4: 239- 244.
15. Speicher KD, Kolbas O, Harper S, Speicher DW. Systematic analysis of peptide Recoveries from In-Gel Digestions for femtomole protein Identifications in Proteome studies. *J Biomol Tech*, 2000; 11:74-86.
16. Hay FC & Westwood OMR. *Practical Immunology*. USA, Blackwell Science. 2002; 163-178.
17. Mikszta JA, Dekker III JP, Harvayl NG, Dean CH, Brittingham JM, Huang J, Sullivan VJ, Dyas B, Roy CJ, Ulrich RG. Micro needle Based intradermal delivery of the anthrax recombinant protective antigen vaccine. *Infect Immun*, 2005; 6806-6810.
18. Wang Y, Kelly CG, Singh M, McGowan EG, Carrara A, Bergmeier LA, Lehner T. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of Heat Shock Protein 70. *J Immunol*, 2002; 169: 2422-2429.
19. Nemoto TK, ONO T, Tanaka K. Substrate-binding characteristics of proteins in the 90kDa heat shock protein family. *Biochem J*, 2001; 354: 663-670.