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# Production and Characterization of Monoclonal Antibodies against the 90-kDa Heat Shock Protein

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**The 90 kDa-heat shock protein (HSP90) is one of the major ubiquitous heat shock proteins induced by a variety of cellular stresses. HSP90 is constitutively synthesized even under nonstressed conditions and found in association with several regulatory and structural proteins such as protein kinases and steroid hormone receptors. In the present study, to facilitate its biochemical characterization, HSP90 was purified from chick muscle by sequential column chromatography steps including DEAE-cellulose, hydroxyapatite, and Sephacryl S-300 gel filtration and monoclonal antibodies specific to HSP90 were produced by the murine hybridomal technique. We report the production of 4 positive hybridoma clones, named as A204, C112, C302 and C410. Among these MoAbs, C112 strongly recognized chick HSP90 in Western blot and native immunoprecipitation. In addition, C112 showed the cross-reactivities against HSP90 from human, rabbit, mouse, fish and chick but not from Drosophila and *E. coli*.**

**KEY WORDS: Monoclonal Antibodies, HSP90**

HSP90 is one of the major heat shock proteins (HSPs) highly expressed in cells in response to a variety of cellular stresses such as elevated growth temperatures, heavy metals, and amino acid analogs (Welch *et al.*, 1991). HSP90 is an abundant cytosolic protein even in unstressed cells and its content is as high as 1-2% of the total cytosolic proteins (Welch *et al.*, 1991). Like other HSPs, HSP90 is ubiquitous and highly conserved during evolution. In mammals and yeast, HSP90 is encoded by two separate genes, whereas in chickens only one form has been identified (Chang and Lindquist, 1994; Hickey *et al.*, 1989; Minami *et al.*, 1991; Moore *et al.*, 1989). The homolog in *E. coli* is called HtpG.

HSP90 has been found complexed with several different regulatory and structural proteins. In higher eucaryotic cells, HSP90 forms complexes with steroid receptors for progesterone, estrogen, glucocorticoid, and androgen and stabilizes the receptors in a non-DNA-binding form until the steroid hormone binds to the receptor (Cadepond *et al.*, 1991; Smith *et al.*, 1993). HSP90 is also reported to interact with tyrosine kinases such as $pp60^{src}$, or serine-threonine kinases including *raf* and *ras* gene products, casein kinase II, eIF-2$\alpha$ kinase, and protein kinase C (Hartson and Matts, 1994; Matts and Hurst, 1989; Wartmann and Davis, 1994; Shi *et al.*, 1994). Thus HSP90 may participate in signal transduction pathways controlled by these protein kinases. Further, HSP90 has been also found associated with cytoskeletal proteins such as actin and tubulin, thereby implicating its role in the regulation of

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cytoskeleton structure in cells (Nishida *et al.*, 1986). HSP90 is regarded to function as a general molecular chaperone and stabilize target proteins in an active, partially unfolded, or unassembled state (Wiech *et al.*, 1992). HSP90 can be post-translationally modified by phosphorylation on serine/threonine residues and possesses ATPase and/or kinase activities (Csermely and Kahn, 1991; Nadeau *et al.*, 1993). These ATPase and/or kinase activities as well as autophosphorylation of HSP90 are suggested to be involved in its interaction with other proteins. However, the exact molecular action mechanism of HSP90 is not clearly understood.

In the present study, in order to facilitate the detection and biochemical characterization of HSP90, we produced monoclonal antibodies (MoAbs) against HSP90 and characterized their specificities and cross-reactivities.

## Materials and Methods

### Cell culture

NS1 myeloma cells and K562 erythroleukemia cells were provided by Aichi Cancer Institute, Nagoya, Japan. HeLa cells, NIH3T3 cells and NS1 myeloma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS) and 1% penicillin/streptomycin (P/S) and K562 erythroleukemia cells grown in Rosewell Park Memorial Institute (RPMI) medium 1640 plus 10% CS and 1% P/S at 37°C in an incubator with 5% $CO_2$. Chick embryonic fibroblasts were obtained as described in Ha *et al.* (1979) and cultured at 37°C in MEM supplemented with 10% CS, 10% chick embryo extract and 1% P/S. Drosophila Kc cells were cultured at 25°C in M3(BF) medium supplemented with 2% heat-inactivated fetal bovine serum (FBS) and 0.5% P/S and Chinook salmon embryonic cells (CHSE-214) grown at 18° C in DMEM supplemented with 10% FBS and 1% P/S.

### Purification of chick HSP90

HSP90 was purified from chick muscle as described in Welch and Feramisco (1982). Chick muscle was homogenized in buffer BT (20mM Tris-acetate, pH 7.4, 20 mM NaCl, 0.1 mM EDTA, and 10 mM 2-mercaptoethanol, and 0.1% Triton X-100) and the soluble proteins were separated from insoluble material by centrifugation at 12,000 ×g for 30 min at 4°C. The supernatant was applied to a DEAE-cellulose ion exchange column (2.5 × 13 cm) equilibrated in buffer BT. After extensive washing of the column with buffer BT, the proteins were eluted with a linear gradient of NaCl (20-300mM) in buffer BT and 80 fractions were collected. The peak fractions of HSP90 (as confirmed by Western blotting with anti-HSP90 antibody (AC88, a gift of Dr. Toft)) eluting off the DEAE column were pooled and applied to an hydroxyapatite column (2.5 × 10 cm) equilibrated in buffer CT (20 mM $K_2HPO_4$, pH7.4, 20 mM NaCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1% Triton X-100). After washing the column with buffer CT, the proteins were eluted with a linear gradient of $K_2HPO_4$ in buffer CT (20-300 mM) and 70 fractions were collected. The peak fractions of HSP90 (confirmed by Western blotting) were pooled and concentrated against buffer BT by ultrafiltration (Amicon 8010). The concentrated proteins, in a volume of 5 ml, were applied to a Sephacryl S-300 gel filtration column (1.3 × 110 cm) equilibrated in buffer BT and fractions were collected. The peak fractions of HSP90 were pooled, dialyzed against phosphate-buffered saline (PBS), and lyophilized by a freeze drier.

### Production of monoclonal antibodies against HSP90

Monoclonal anti-HSP90 antibody was produced according to the procedure of Kennett (1988). Briefly, HSP90 (70 μg) was injected in complete Freund's adjuvant into Balb/c mouse and boost immunized in incomplete Freund's adjuvant three times at three-week intervals and thereafter the final injection was given without adjuvant. After the final injection, spleen cells were obtained from Balb/c and fused with NS1 mouse myeloma cells in polyethylene glycol (PEG 1000) in DMEM, pH 8.0-8.2. The fused cells were placed in 96 well plates in HT media ($1 \times 10^{-4}$ M hypoxanthine, $1.6 \times 10^{-3}$ M thymidine) containing 20% FBS.

On the following day, HAT media (HT media containing $4 \times 10^{-7}$ M aminopterin) were added on the feeder layer. The produced hybridoma cells were cultured in DMEM plus 20% FBS and 1% P/S and subcloned by limiting dilution and successively in semi-solid agarose.

### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was carried out as described in Kennet (1988). HSP90 was plated onto 96 well plates and after washing three times in PBS/0.05% Tween 20, hybridoma culture media was added and the plates were incubated for 2 h at 37°C. After washing, goat anti-mouse IgG-peroxidase conjugate was added. After extensive washing in PBS/0.05% Tween 20, 20 $\mu$l of substrate solution (10 mg 0-phenylenediamine, 4 $\mu$l of 30% $H_2O_2$ solution in 10ml of 0.1 M citrate buffer, pH 4.5) was added and the absorbance was measured at 405 nm with a multiscan photometer (Titertek Multiskan, Flow Laboratories).

### SDS-Polyacrylamide gel electrophoresis and western blot analysis

Cultured cells were washed in cold PBS and lysed in SDS-Laemmli sample buffer by heating at 100°C for 10 min. Tissues were homogenized in buffer BT and the homogenate was adjusted to SDS-Laemmli sample buffer condition and boiled at 100°C for 10 min. The protein content for each sample was quantified by using bicinchoninic acid protein assay kit (Sigma Chemical Co.). Equal amounts of proteins (50 $\mu$g) were separated by one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The resulting gels were either stained with Coomassie Blue or transferred to nitrocellulose paper. For Western blotting (Towbin *et al.*, 1979), the membrane was blocked with 5% nonfat milk in Tris buffered saline/0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were then incubated with antibody for 1-2 h at room temperature. After washing in TBS-T three times, the blot was incubated with alkaline phosphatase- or peroxidase-conjugated secondary antibody and the antibody-specific proteins were visualized either by NBT/BCIP staining method or by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp.).

### Immunoprecipitation

For the native immunoprecipitation, labeled cells were solubilized in RIPA(–) buffer (1% Triton X-100, 1% deoxycholate in PBS). In case of denaturing immunoprecipitation, labeled cells were lysed in SDS-Laemmli sample buffer by heating at 100°C for 10 min and then adjusted to RIPA(–) condition. The cell lysate were preabsorbed in Sepharose CL-4B at 4°C for 30 min and the resulting supernatant incubated with antibody at 4°C for 2 h. The immune complexes were captured by protein A-agarose beads and the beads were washed with RIPA(–) 5 times. The proteins were then released by boiling in SDS-Laemmli sample buffer and analyzed by SDS-PAGE and fluorography.

## Results and Discussion

### Purification of HSP90

To facilitate its biochemical characterization and for the production of monoclonal antibodies, HSP90 was purified from chick muscle by sequential column chromatography steps including DEAE-cellulose, hydroxyapatite, and Sephacryl S-300 gel filtration. Chick muscle homogenate in buffer BT were clarified of insoluble materials by centrifugation at 12,000 $\times$g for 20 min and the supernatant was applied to a DEAE-cellulose column. The proteins were eluted with a linear gradient of NaCl (20-300 mM) (Fig. 1A) and the peak fractions of HSP90 were pooled and separated by hydroxyapatite chromatography (Fig. 1B). The peak fractions of HSP90 were pooled and further purified by Sephacryl S-300 gel filtration (Fig. 1C). As shown in Fig. 2, the purified HSP90 proved to be essentially homogeneous as judged by SDS-PAGE and Western blotting using anti-HSP90 antibody (AC88).

### Production and characterization of monoclonal antibodies against HSP90

To produce MoAbs against HSP90, Balb/c

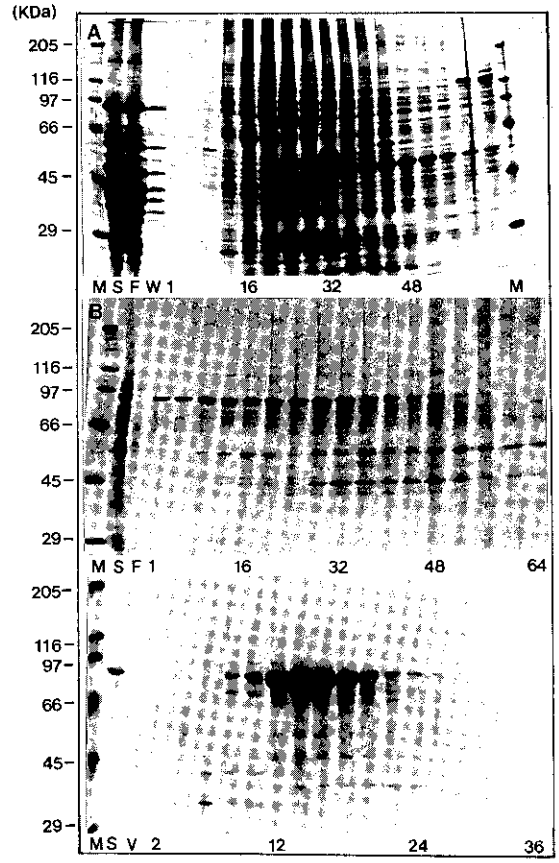


**Fig. 1.** Purification of HSP90 from chick muscle. *Panel A.* Chick muscle homogenate in buffer BT was centrifuged at 12,000 ×g for 30 min and the resulting supernatant was fractionated by DEAE-ion exchange chromatography and an aliquot of every fourth fraction was analyzed by SDS-PAGE. Shown is the Coomassie blue stained gel. Lane M represents molecular weight marker proteins; lane S, the material applied; lane F, the flow through; lane W, the material eluted by wash with buffer BT. *Panel B.* The peak fractions of HSP90 eluting off the DEAE column (fractions 25 to 72) were pooled and applied to a hydroxyapatite column. An aliquot of every fourth fraction was analyzed by SDS-PAGE. Lane M represents molecular weight marker proteins; lane S, the material applied; lane F, the flow through. *Panel C.* The peak fractions (fractions 24 to 62) of HSP90 eluting off the hydroxyapatite column were pooled, concentrated and applied to a Sephacryl S-300 column. An aliquot of fractions was analyzed by SDS-PAGE. Lane M represents molecular weight marker proteins; lane S, the material applied; V, the void eluant.

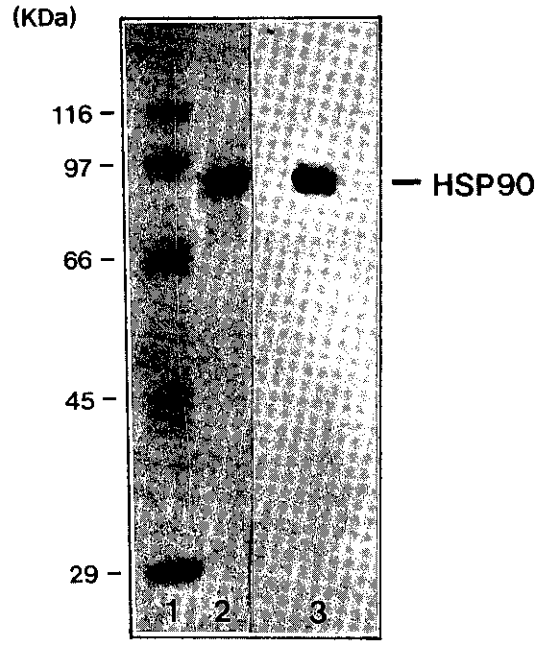


**Fig. 2.** Identification of purified HSP90 by Western blotting with AC88. HSP90 purified as described in Fig. 1 was analyzed by SDS-PAGE and then either Coomassie blue-stained (lanes 2) or Western blotted with AC88 (lane 3). Lane 1 represents molecular weight marker proteins.

mouse was immunized with purified HSP90. The spleen cells were prepared from Balb/c and fused with NS-1 myeloma cells, and the fused hybridoma cells were then grown in HAT medium as described in Materials and Methods.

From eight 96 well-plates, we initially obtained 24 positive clones as determined by ELISA and the hybridoma clones were further subcloned by limiting dilution and successively in semi-solid agarose.

Western blot analysis was carried out in order to test the specificities of these hybridoma clones. Among 24 ELISA-positive hybridoma clones, 4 clones (named as A204, C112, C302 and C410) showed positive reactions on the Western blot. As shown in Fig. 3, C112 recognized HSP90 strongly than A204, C302 and C410. Since C112 exhibits the great reactivity to HSP90, its properties were further characterized. C112 was of $IgG_{2a}$ isotype as judged by mouse MoAb isotyping kit.

To further characterize the specificity of the MoAb C112, immunoprecipitation was performed. For the preparation of labeled HSP90,

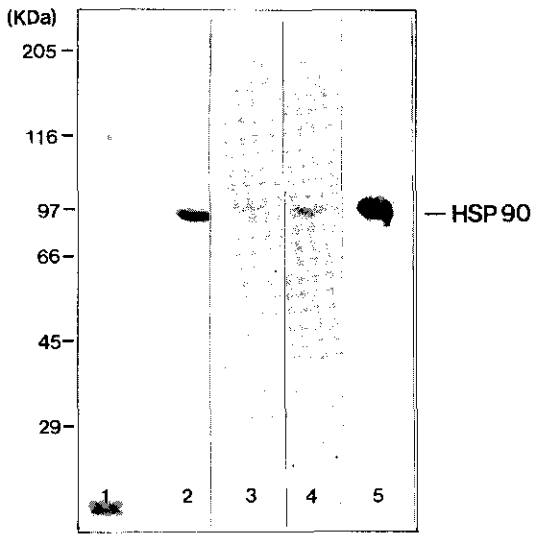


**Fig. 3.** Western blot analysis of the monoclonal antibodies. The purified HSP90 was analyzed by SDS-PAGE and the resulting gels were Coomassie blue-stained (lanes 1-2) or Western blotted with hybridoma cell culture media (lanes 3-5). Lane 1 represents molecular weight marker proteins. Lane 2, Coomassie stained HSP90; lane 3, MoAb C302; lane 4, MoAb C410; lane 5, MoAb C112.

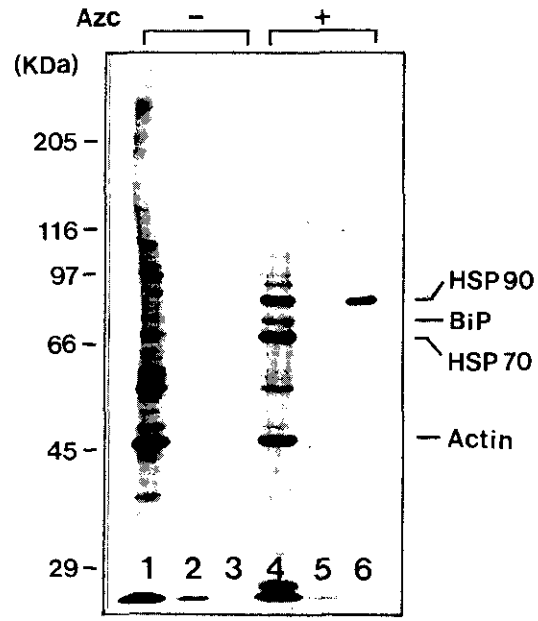


**Fig. 4.** The immunoprecipitation analysis with the MoAb C112. Chick fibroblast cells were preincubated in the absence (-) or presence (+) of 5 mM L-azetidine-2-carboxylic acid (Azc) for 4 h and labeled in $^{35}$S-methionine for 3 h. The labeled cells were harvested in RIPA (-) and immunoprecipitation was performed with preimmune serum (lanes 2 and 5) or with C112 (lanes 3 and 6) and analyzed by SDS-PAGE and fluorography. Lanes 1 and 4, the labeled total cell lysates.

chick embryonic fibroblasts were preincubated in the absence and presence of 5 mM L-azetidine-2-carboxylic acid, a proline analog and one of strong HSP inducers, for 4 h and labeled in $^{35}$S-methionine for 3 h. The labeled cells were lysed either in SDS-Laemmli sample buffer or in RIPA(-) as described in Materials and Methods and immunoprecipitated with the MoAb C112. As shown in Fig. 4, the exposure of cells to L-azetidine-2-carboxylic acid highly induced the syntheses of HSPs including HSP90, HSP70 and BiP/GRP78. In the denaturing immunoprecipitation, C112 did not immunoprecipitate HSP90 (data not shown). In case of the native immunoprecipitation, the MoAb C112 immunoprecipitated HSP90 in normal cells as well as in L-azetidine-2-carboxylic acid-treated cells (Fig. 4). In addition, in stressed cells some of cytosolic HSP70 was shown to co-precipitate along with HSP90. This result may suggest the physical association between HSP90 and HSP70. Several studies have indicated that in eucaryotic cells HSP90 exists interacted with HSP70 and other cellular proteins (Czar *et al.*, 1994; Inanobe *et al.*, 1994; Perdew 1988). For example, HSP70 is found associated with complexes of steroid hormone receptor and HSP90 (Hutchison *et al.*, 1992; Sanchez *et al.*, 1990)

Since HSP90, like other HSPs, is known to be highly conserved from procaryotes to higher eucaryotes (Ashburner and Bonner, 1979; Lindquist 1986), the cross-reactivities of these MoAbs with HSP90 from different organisms were examined by Western blot analysis. As shown in Fig 5, in addition to chick HSP90, C112 recognized HSP90 from human, rabbit, mouse, fish but not from Drosophila and *E. coli*. Thus, the MoAb C112 can be used for the identification and characterization of HSP90 that is widely expressed in various organisms as well as in studying its interaction with other cellular proteins.

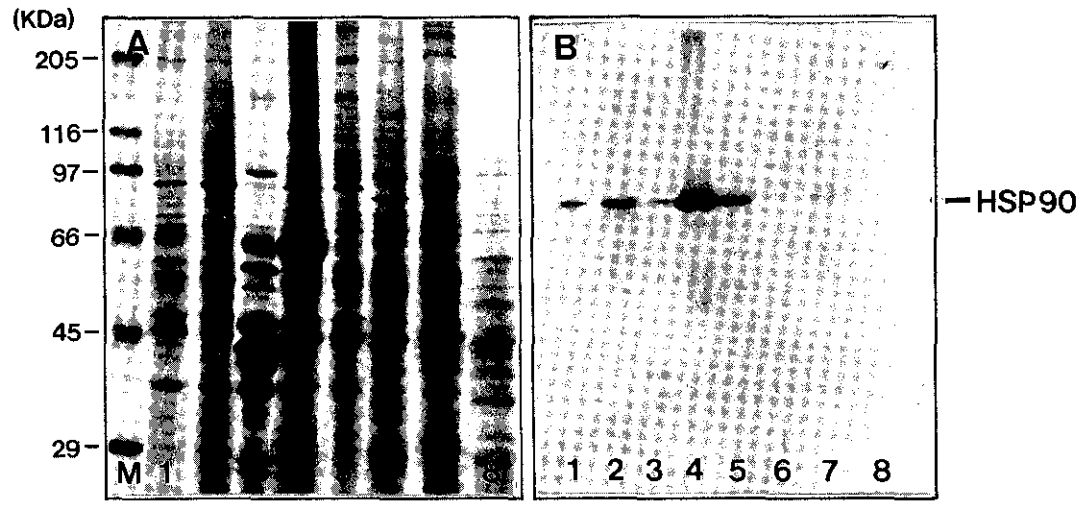


**Fig. 5.** The cross-reactivities of the MoAb C112 with HSP90 from different organisms. The proteins from various organisms were analyzed by SDS-PAGE and the gels were Coomassie-stained (panel A) or Western blotting with the MoAb C112 (panel B). Lane 1, HeLa cells; lane 2, K562 cells; lane 3, rabbit muscle; lane 4, chick fibroblast cells; lane 5, NIH 3T3 mouse cells; lane 6, *Drosophila* Kc cells; lane 7, CHSE-214 salmon cells; lane 8, *E. coli*; lane M, molecular weight marker proteins.

## Acknowledgements

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## HSP90에 대한 단일클론항체의 생성 및 특성 조사

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90kDa-heat shock protein(HSP90)은 여러 가지 스트레스에 의해서 유도되는 주요 스트레스단백질 가운데 하나이다. HSP90은 스트레스가 없는 정상적인 상황에서도 일정량 합성되는 단백질로, 세포 내에서 kinases나 스테로이드호르몬 수용체와 같은 여러 조절단백질 및 구조 단백질과 결합하는 것으로 알려져 있다. 본 연구에서는 HSP90의 생화학적인 특성을 조사하는데 사용할 목적으로 DEAE-cellulose chromatography, hydroxyapatite chromatography, Sephacryl S-300 gel filtration의 방법으로 닭 근육조직으로부터 HSP90을 순수 분리하고, 이에 대한 단일클론항체를 murine hybridoma technique을 이용하여 생성하였다. 생성된 클론들에 대해서 ELISA와 Western blot을 실시한 결과 HSP90을 인지하는 A204, C112, C302, C410의 4가지 단클론을 얻었다. 특히 C112는 Western blot과 native immunoprecipitation 실험에서 인간, 토끼, 닭, 쥐, 어류의 HSP90을 인지하지만 초파리, *E. coli*의 HSP90은 인지하지 못하는 것으로 나타났다.