

Production and characterization of polyclonal antibody against a synthetic peptide from β -actin protein

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ABSTRACT

Objective(s): Antibodies against actin, as one of the most widely studied structural and multifunctional housekeeping proteins in eukaryotic cells, are used as internal loading controls in western blot analyses. The aim of this study was to produce polyclonal antibody against a synthetic peptide derived from N-terminal region of β -actin protein to be used as a protein loading control in western blot and other assay systems.

Materials and Methods: A synthetic peptide derived from β -actin protein was designed and conjugated to Keyhole limpet hemocyanin (KLH) and used to immunize a white New Zealand rabbit. The antibody was purified from serum by affinity chromatography column. The purity of the antibody was determined by SDS-PAGE and its ability to recognize the immunizing peptide was measured by ELISA. The reactivity of the antibody with β -actin protein in a panel of different cell lysates was then evaluated by western blot. In addition, the reactivity of the antibody with the corresponding protein was also evaluated by Immunocytochemistry and Immunohistochemistry in different samples.

Results: The antibody could recognize the immunizing peptide in ELISA. It could also recognize β -actin protein in western blot as well as in immunocytochemistry and immunohistochemistry.

Conclusion: Our data suggest that this antibody may be used as an internal control in western blot analyses as well as in other immunological applications such as ELISA, immunocytochemistry and immunohistochemistry.

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Introduction

Actin is one of the most widely studied structural and multifunctional proteins in eukaryotic cells (1, 2). It has important roles in many cell functions such as maintenance of cytoskeleton, cell motility, adhesion, endocytosis, exocytosis, secretion, signal transduction, and other activities (3-5). Among its three main isoforms (α , β and γ), α -actin is normally found in smooth muscle cells whereas β - and γ -actin isoforms are found in all cells (6). Antibodies against β -actin and other housekeeping gene-encoded proteins, like β -tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are used as internal loading controls in western blot analyses (7, 8).

The aim of this study was to produce polyclonal antibody against a synthetic peptide derived from N-terminal region of β -actin protein to use as a protein

loading control in western blot and other assay systems.

Materials and Methods

Design of peptide and conjugation to carrier protein

A 12-mer peptide (DDDIAALVVDNG) from the N-terminal region of β -actin protein was designed. The immunograde peptide was purchased (Thermo Electron Corporation, GmbH, Ulm, Germany) and then conjugated to Keyhole limpet hemocyanin (KLH) and Bovine serum albumin (BSA) (Sigma, St. Louis, MO) via glutaraldehyde as linker separately and simultaneously using the same buffer systems and methods as described (9). The peptide-KLH conjugate was used for rabbit immunization and the peptide-BSA was used for conjugation efficacy assessment.

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Confirmation of conjugation by SDS-PAGE

To check the efficacy of conjugation, 10 μ g of peptide-BSA was run on 10% SDS-PAGE using a mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, Philadelphia, PA). The gel was stained with Coomassie Blue R-250 (Sigma). The BSA-peptide conjugate was used to test the conjugation efficacy, since the KLH-conjugate was a very large protein conjugate to enter the separating gel during electrophoresis, and thus impossible to be evaluated by SDS-PAGE directly.

Rabbit immunization

A female white New Zealand rabbit was immunized 5 times with two-week intervals for each injection. In the first immunization, 250 μ g KLH-peptide and an equal volume of Freund's complete adjuvant (Sigma) were mixed and injected intramuscularly into the femoral muscle. For the subsequent immunizations, 125 μ g peptide-KLH was emulsified in Freund's incomplete adjuvant (Sigma) and injected.

Titration of antibody in serum samples

Before each immunization, blood was drawn by venous puncture from the rabbit ear and allowed to clot for 2 to 3 hr at room temperature before preparation of sera. Titration of the specific polyclonal antibody was then performed using ELISA (10).

Antibody purification

Rabbit sera were filtered through 0.45 μ m filters and antibody was purified by affinity chromatography column prepared by coupling the immunogenic peptide to CNBr-activated sepharose 4B (GE Healthcare, Uppsala, Sweden). The recovery of the antibody, the evaluation of its reactivity with immunizing peptide and the assessment of its purity were performed as described previously (10).

Cell lysate preparation and western blot analysis

The ability of the antibody to recognize β -actin was assessed by western blotting. Different samples were collected and each was lysed in 1 ml of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% protease inhibitor cocktail (Sigma) for 1 hr on ice with 15 min intervals of vortexing for 30 sec. The cell lysates were then centrifuged at 500 \times g for 30 min. The supernatants were collected and protein concentrations in the lysates were measured by BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Scientific, IL, USA). Twenty μ g of each sample was run on a 10% SDS-PAGE (100 V for 2 hr) under both reduced and non-reduced conditions. After electrophoresis, the resolved proteins were transferred onto Immobilon-PVDF membranes (Millipore Corporation, USA). The

membranes were blocked overnight at 4°C with 5% non-fat milk in PBS containing 0.05% Tween 20 (PBS-T). All antibody incubations were performed in PBS-T containing 3% non-fat milk. Filters were incubated with 10 μ g/ml of anti- β -actin antibody for 1.5 hr at room temperature. After extensive washing with PBS-T, the filters were incubated with peroxidase-conjugated sheep anti-rabbit immunoglobulins (Avicenna Research Institute, Tehran, Iran) for 1 hr at room temperature followed by washing and developing with ECL chemiluminescent detection system (GE Healthcare, Uppsala, Sweden). Negative controls were used to check the probable interaction with secondary antibody (HRP-conjugated sheep anti-rabbit antibodies), which included all the above-mentioned steps except for adding the anti- β -actin antibody to the membranes.

In order to determine the minimum concentration of lysate proteins that could show a clear band in western blot with the antibody, a gradient of protein concentrations (0.5-20 μ g) of Jurkat cell line lysate were used in the assay under non-reduced conditions.

Assessment of antibody reactivity using immunocytochemistry (ICC) and immunohistochemistry (IHC) assays

Three cell populations including peripheral blood mononuclear cells (PBMC) as well as CHO and Jurkat cell lines were selected for Immunocytochemistry assays. PBMC from a healthy blood donor were separated by Ficoll density gradient centrifugation (11). CHO and Jurkat cell lines (ATCC, USA) as well as freshly-prepared PMBC were cultured (2-3 \times 10⁴ cells/well) on 8-well laminated glass slides (Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany) in RPMI 1640 containing 10% FBS (Invitrogen, Carlsbad, CA) with subsequent incubation in a CO₂ incubator overnight. In addition, acetone-fixed cryostat sections from three different samples (Crab, Gold fish, and Neon fish) were prepared for immunohistochemistry assay. The slides were then immunostained as described in previous research (12) and were examined under a fluorescent microscope (Olympus, Tokyo, Japan).

Results

Confirmation of conjugation by SDS-PAGE

The quality of conjugation of the peptide to the carrier protein KLH was assessed by running peptide-BSA conjugate on SDS-PAGE. Change in mobility shift of conjugated BSA compared to non-conjugated BSA confirmed the efficiency of conjugation (data not shown).

Antibody titration

Presence of antibody against the immunizing peptide in rabbit sera was assessed by ELISA before

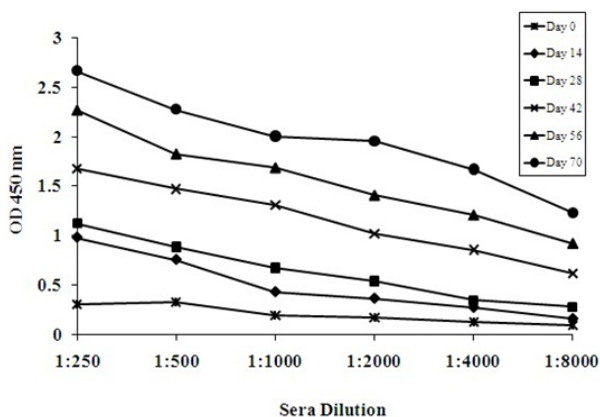


Figure 1. Kinetic evaluation of anti- β -actin antibody production in serum samples of the immunized rabbit. A white New Zealand rabbit was immunized 5 times with peptide-KLH conjugate. The reactivity of diluted sera (serial dilutions from 1:250 to 1:8000) from the immunized rabbit was determined at different time intervals by ELISA

and after immunization. As shown in Figure 1, the antibody titer in the rabbit serum continuously increased upon repeated immunizations.

Evaluation of purification

SDS-PAGE analysis of the purified antibody showed the presence of a single band of about 150 kDa indicative of the desired purity (Figure 2). ELISA assay showed that the antibody had excellent immunoreactivity with the immunizing peptide, confirming its functionality after purification. In addition, lack of reactivity of the antibody with an irrelevant peptide and BSA, as an irrelevant protein, confirmed its specificity for the immunizing β -actin peptide (Figure 3).

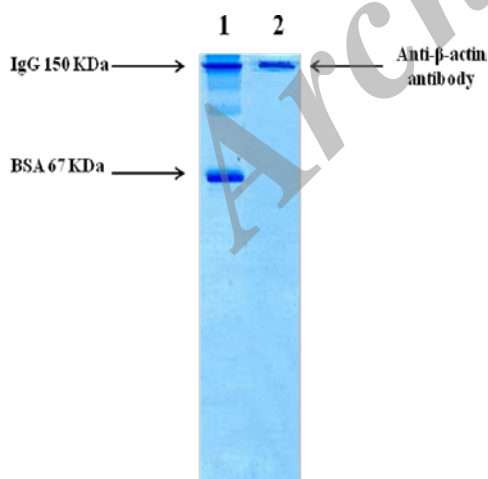


Figure 2. Analysis of the purified anti- β -actin antibody by SDS-PAGE. Polyclonal anti- β -actin antibody was produced in rabbit and purified over peptide affinity column. The purity of the antibody was assessed by SDS-PAGE. Lane 1: A mixture of human IgG and Bovine serum albumin as protein marker. Lane 2: Purified antibody

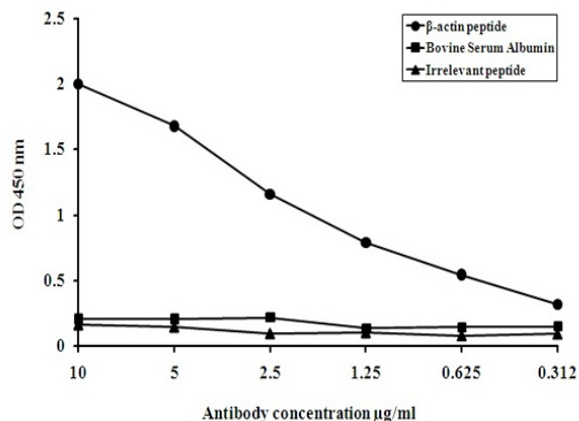


Figure 3. Titration of the purified rabbit anti- β -actin antibody. Anti- β -actin antibody was purified by peptide affinity column and its reactivity with the immunizing peptide was evaluated by ELISA. Lack of reactivity of the antibody with Bovine serum albumin and a 19-mer irrelevant peptide that served as negative controls are also presented

Western blotting

To determine the reactivity of the purified polyclonal antibody to its corresponding protein, western blot assay was performed using samples from different origins. The antibody could recognize a band of about 45 kDa, representative of β -actin protein in all samples under reducing-conditions except for Coral and Crab (Figure 4A). The antibody could also interact with most samples being included under non-reducing conditions except for HeLa, Rose Aphid, Coral, Shrimp, Crab and Drosophila (Figure 4B). Furthermore, western blot on a gradient of different amounts (0.5 to 20 μ g) of Jurkat cell lysate proteins revealed that the antibody could interact with β -actin content of a minimum amount of 2.5 μ g of total proteins in the lysate (Figure 5).

Immunocytochemistry (ICC) and immunohistochemistry (IHC) assays

Immunofluorescent assays were performed using anti- β -actin antibody and six randomly selected samples. The green fluorescence is indicative of the interaction of the antibody with β -actin in all samples (CHO, Jurkat and PBMC) in ICC (Figure 6A) as well as (Crab, Gold fish and Neon fish) in IHC (Figure 6B). The blue color represents the staining of nuclei by DAPI.

Discussion

Western blot analysis is a widely used semi-quantitative method for specific determination of protein expression (13). When a negative sample is encountered in western blot, it may be interpreted as negative. However, to ensure that the result is not a false negative due to sample preparation errors or other experimental faults, it is necessary to have appropriate controls. Although there are some reports regarding different expression profiles of the

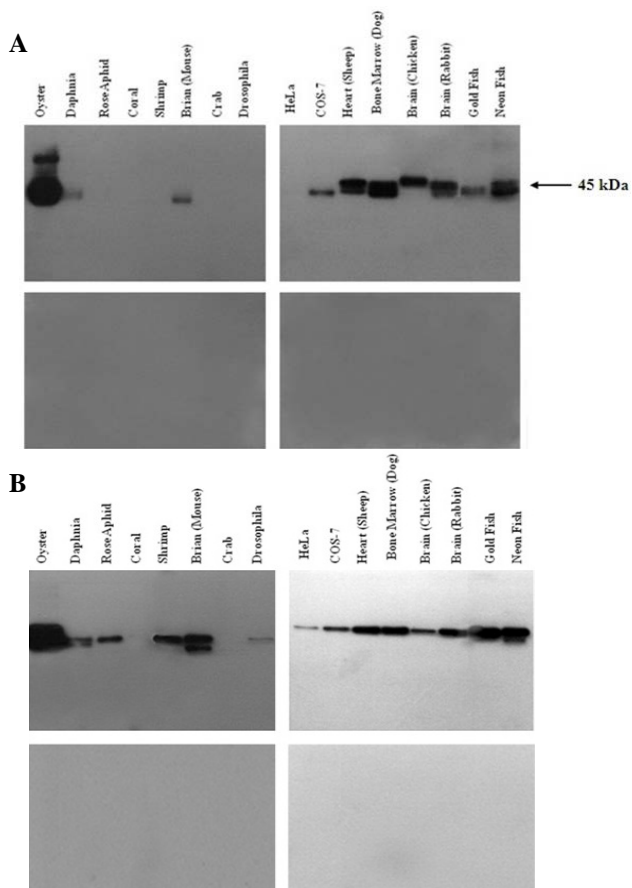


Figure 4. Analysis of reactivity of anti- β -actin antibody with lysates from a panel of organisms with different origins by western blot. A panel of tissue samples from different organisms were lysed and subjected to western blot under reducing (A) and non-reducing (B) conditions. The lower panels are negative controls where no anti- β -actin antibody was used

housekeeping proteins in animal and experimental models (14, 15), housekeeping proteins are extensively used to confirm the quality of the experiments(16, 17). One of the most widely used housekeeping proteins in western blot is β -actin. In order to facilitate its use for normalizing protein contents in western blot experiments, a suitable antibody for its detection is highly needed.

In this study, we produced and characterized a polyclonal antibody against a synthetic peptide derived from β -actin protein. The peptide sequence encompasses a conserved region of β -actin expressed by many (but not all) organisms with different origins. Interestingly, the antibody did not react with lysates from Coral and Crab, while it showed reactivity with that from the other organisms. This may be because the antibody was made against a selected peptide from human β -actin and its lack of reactivity with Coral and Crab might be a consequence of the absence of the antibody epitope on the actin molecules from these organisms, which may in turn be due to differences in amino acid sequences, post translational modifications or differential RNA splicing events. This was also confirmed when amino acid sequences of the

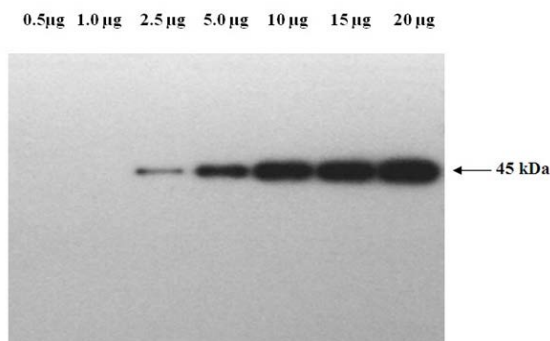


Figure 5. Western blot assay on different concentrations of Jurkat cell lysates. Different amounts of Jurkat lysate proteins (0.5 to 20 μ g) were run on SDS-PAGE gel under non-reducing conditions followed by blotting and detection with anti- β -actin antibody

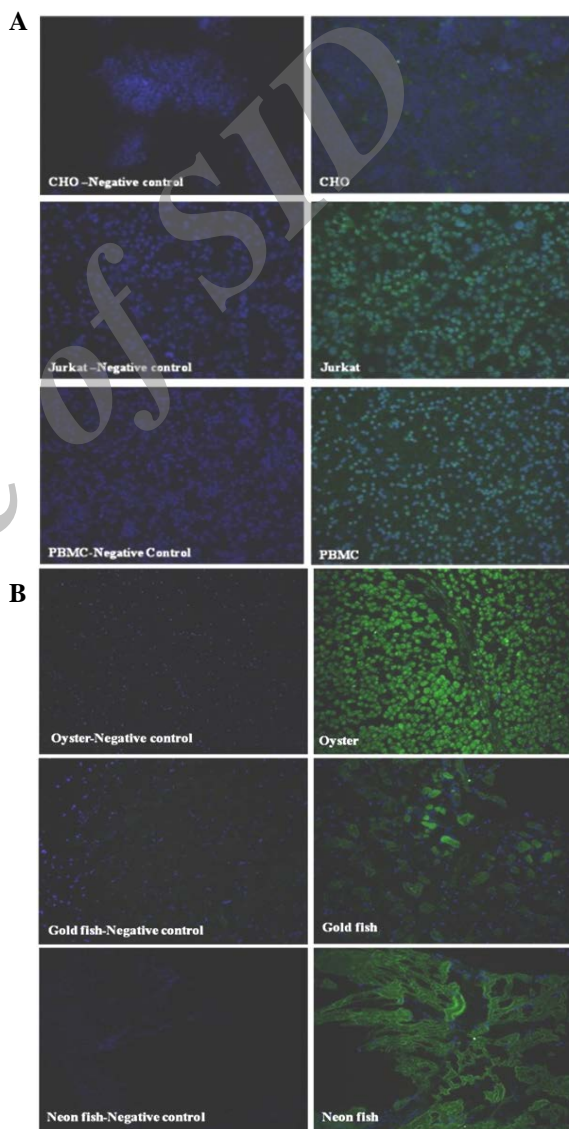


Figure 6. Immunocytochemistry (ICC) and immunohistochemistry (IHC) assays using anti- β -actin antibody. FITC-conjugated sheep anti-rabbit antibody was used as secondary antibody. DAPI was used for staining the nuclei. Anti- β -actin antibody was used to detect β -actin in CHO, Jurkat and PBMC using ICC (A) and in Crab, Gold fish and Neon fish using IHC (B). Figures on the left panels in both A and B are the negative controls where no anti- β -actin antibody was used

β -actin molecules from such organisms were compared in protein blast database (<http://blast.ncbi.nlm.nih.gov>) where differences were observed (data not shown).

Differential reactivity of the antibody under reducing and non-reducing conditions may be explained by the presence of disulfide bridges in the protein molecule that may hide the epitopes from antibody access. Reducing agents such as 2-mercaptoethanol (2-ME) may expose the hidden epitopes by breaking these bridges. Our data may thus suggest that reducing conditions are preferable because a wider range of organisms can be recognized as compared to non-reducing conditions.

Conclusion

Although the main aim of this study was to produce an antibody against β -actin to be used as internal control in western blot analyses, our data suggest that the antibody may be used in other immunological applications such as ELISA, Immunocytochemistry and immunohistochemistry.

Acknowledgment

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