A Recombinant GST-EMBP440 Fusion Protein from Sea Urchin Embryos that has Myosin Binding Capabilities

by

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Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in the
Biological Sciences
Program

SCHOOL OF GRADUATE STUDIES
YOUNGSTOWN STATE UNIVERSITY
August, 1997

A RECOMBINANT GST-EMBP 440 FUSION PROTEIN FROM SEA URCHIN EMBRYOS THAT HAVE MYOSIN-BINDING CAPABILITIES

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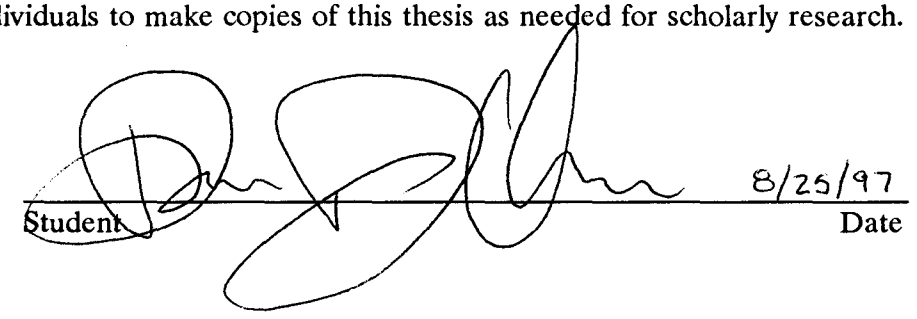

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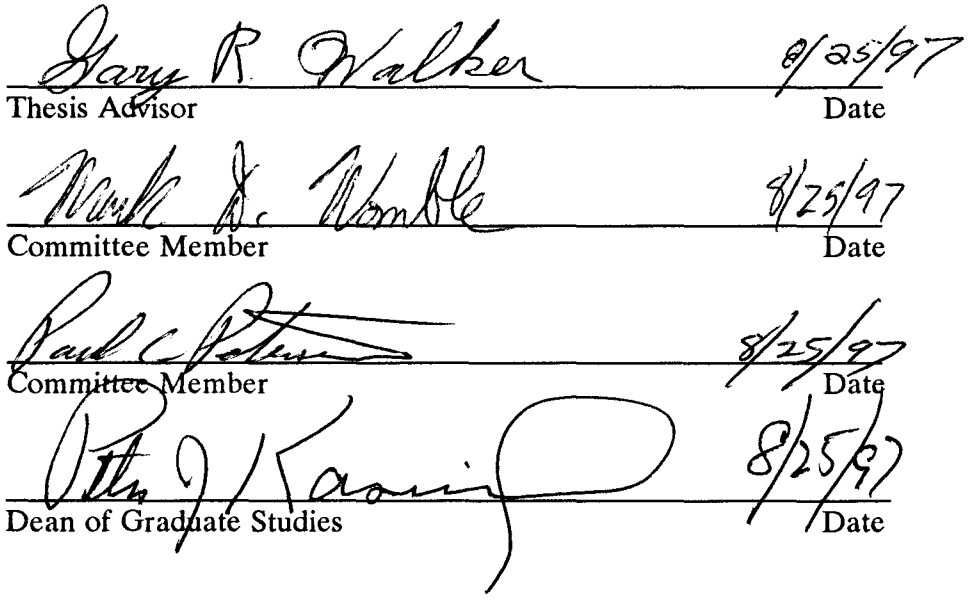

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I. Abstract

A 53 kDal. (53K) native protein was isolated from sea urchin egg extract. The properties of this protein suggests that it may be a titin-like protein. Previous studies have shown that this protein has myosin light chain kinase activity. The antibody to native 53 kDal. was used to select recombinant GST-EMBP440 protein from a cDNA library. GST-EMBP440 protein was used in this study. This study confirms the myosin binding activity of GST-EMBP440 protein. Two binding assays were utilized to demonstrate myosin binding by GST-EMBP440 protein. A blot binding assay utilizing immunodetection suggested a lack of 53 kDal. binding. Results from a glutathione-agarose affinity precipitation assay indicated binding of the GST-EMBP440 fusion protein to myosin. The findings therefore suggest GST-EMBP440 protein may indeed be a myosin binding protein.

Acknowledgments

I would like to express my sincere gratitude to Dr. Gary Walker for his guidance, suggestions and encouragement throughout the preparation of this thesis.

I would also like to thank Dr. Paul Peterson and Dr. Mark Womble for serving on my committee.

Finally, I would like to thank my family and Colleen Cunningham for their patience and support while I worked on this project.

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II. Introduction

Myosin is a molecular motor protein of great importance in many cell types. It comprises nearly 50% of the total protein in skeletal muscle (Harrington and Rogers, 1984). Myosin is also found in non muscle cells of vertebrate and invertebrate animals, in higher plants, and in unicellular organisms.

Myosin II contributes to movements ranging from muscle contraction to cytokinesis in non-muscle cells (Kiehart, 1990). It is hexmeric with two heavy chains and four light chains. The pair of heavy chains form two amino-terminal globular heads that contain the ATP-binding sites to which the actin filaments bind to when activated by Mg^{2+}-ATPase activity (Korn and Hammer, 1988). The remainder of the heavy chain forms an alpha-helix with a rod-like tail through which the molecules associate into bipolar filaments under low ionic conditions (Korn and Hammer, 1988). High salt has been demonstrated to change the conformation of myosin's tail (Trybus and Lowey, 1984). The bipolar filaments interact with actin filaments much in the same way as muscle myosin thick filaments. This is carried out by the cross-bridge

cycling of the globular heads that moves the actin filaments in opposite orientation to the heads at the two ends of the myosin filaments (Korn and Hammer, 1988). Myosin heavy chains alone are likely to be responsible for chemomechanical force production, whereas the light chain functions to modify and regulate myosin heavy chain activity (Kiehart, 1990). The myosin light chains can be phosphorylated by myosin light chain kinase and protein kinase C. This effects the ATPase activity of myosin (Higashihara, Takahata, Kurokawa, 1990). Light chain phosphorylation promotes bipolar filament assembly, whereas heavy chain phosphorylation inhibits bipolar filament assembly (Yabkowitz and Burgess, 1987).

Myosin II and actin become localized to the contractile ring of dividing cells that results in the cleavage furrow formation and cytokinesis (Mabuchi, 1994). Actin filaments uniformly are distributed in the cortex at metaphase and form a meshwork over the entire cortex at anaphase (Mabuchi, 1994). After the equatorial alignment of actin filaments, myosin heavy chains become dephosphorylated and myosin assembles at the equator during anaphase (Fishkind and Wang, 1995). Therefore, phosphorylation is partially responsible

for regulating myosin's interaction with actin (Fishkind and Wang, 1995).

Regulation of cellular activities such as cytokinesis depends on the state of polymerization of both actin and myosin (Yabkowitz and Burgess, 1987). Actin can exist in many forms such as G-actin, F-actin, and in complex forms where its polymerization and organization dependent on interactions with actin-binding proteins (Pollard and Cooper, 1986).

Myosin also needs to assemble into insoluble filamentous arrays and disassemble for cellular activities such as cytokinesis to occur. Myosin binding proteins regulate myosin filament structure (Keller, 1995). Some examples of these myosin binding proteins are C-protein, M-protein, myomesin and titin. C-proteins regulate and stabilize thick filaments during the assembly of sarcomeric A-bands (Ward, Lemanski, Unaltuna and Dube, 1995). The carboxyl terminus in particular of the C-protein specifies its incorporation into the A-band of striated muscle (Gilbert, Kelly, Mikawa and Fischman, 1996). In non-muscle cells, the C-protein forms spindle-shaped structures arranged in bundles of myosin thick filaments (Seiler, Fischman and Leinward, 1996). The M-protein is a structural protein localized in the M-band of

muscle and is restricted to fast twitch fibers. Myomesin, like M-protein, is a structural protein localized to the M-band of muscle. Unlike M-protein, myomesin is present in both fast and slow twitch fibers (Obermann, Gautel, Steiner, Van der Ven, Weber and Fuerst, 1996).

The myosin binding protein, titin, is of particular interest. Titin can be found in both vertebrate and invertebrate muscles where their structure and regulation may vary. Vertebrate titin span half of a muscle sarcomere of the thick filaments during contraction and generate passive tension through elastic extension when sarcomeres are stretched. Invertebrate titins are confined to the A-band of the sarcomere and may play more of a regulatory role in sarcomere function. Therefore, the properties of various titins range from primarily structural to regulatory where phosphorylation and kinase activities are seen (Keller, 1995). Titin molecules could serve as a template for filament assembly causing the alignment of filaments into highly ordered arrays (Eilertsen, Kazmierski and Keller, 1994). Titin may also be an early marker of myogenic differentiation both in vivo and in vitro (Van der Loop, Van Eys, Schaart and Ramaeker, 1996).

Titin interacts with other myosin binding proteins. The M-band proteins, myomesin and M-protein, interact with the carboxy terminal region of titin to coordinate the assembly of thick filaments (Obermann, Gautel, Weber and Fuerst, 1997). The C-protein also interacts with titin at the C-region of thick filaments during their assembly (Freiburg and Gautel, 1996). Titin itself forms an elastic link to the Z-line, which maintains alignment of the thick filaments and resists overstretching of the sarcomere (Eilertsen, Kazmierski and Keller, 1994).

In sea urchin egg extracts, a protein (EMBP53K) of a molecular mass of 53 kilodaltons co-precipitates with myosin upon the addition of nucleoside-triphosphate (Yabkowitz and Burgess, 1987). The protein mediates the low ionic strength solubility of myosin (Yabkowitz and Burgess, 1987). The binding of the 53K protein to myosin appears to be dependent upon nucleoside-triphosphate and is also sensitive to high ionic strength (Yabkowitz and Burgess, 1987). The heavy chain head-rod junction is the region on myosin where the 53K protein binds (Walker, Yabkowitz and Burgess, 1991).

A lambda zap cDNA phage library derived from sea urchin eggs was screened using anti-53K(EMBP)

to get 8 clones. Through the use of subcloning and screening, two pure clones were made and placed into Bluescript plasmid. A 440 base pair Bluescript insert was then subcloned into pGEX-3X, resulting in an inducable GST-EMBP fusion protein(EMBP440). The glutathione sepharose transferase fusion protein is capable of being purified from isopropyl-thio-glactopyranocide (IPTG) induced bacterial lysates by this method (Walker, unpublished data).

The object of this study is to look at the myosin binding characteristics of this recombinant fragment of the 53K protein (GST-EMBP440). Previous studies have shown that this protein has myosin light chain kinase activity (Walker, unpublished data). This suggested that this may be a myosin binding-protein. In addition, antibodies raised against GST-EMBP440 recognize a very large protein (~1-2000 kDal.) in rabbit skeletal muscle by immuno-blot analysis. This suggests GST-EMBP440 may be related to titin like molecules (Walker, unpublished data). To further show that this may indeed be a myosin-binding protein, the present study demonstrates directly the myosin-binding activity associated with this protein. Binding assays were utilized to demonstrate myosin binding by this protein. This was accomplished in two

ways: a blot binding assay and glutathione-agarose affinity precipitation assay.

III. Materials and Methods

pGEX-EMBP440 Plasmid:

The recombinant pGEX-EMBP440 plasmid is derived from pGEX plasmid (Pharmacia, Inc.) that has a glutathione-S-transferase (GST) gene which is under the control of a lac promoter. The C-terminal end of the GST gene has a ECoR1 restriction site. The insertion point that codes for EMBP440 use this ECoR1 restriction site. The plasmid has a ampicillin resistance gene to allow for the selection of the plasmid-containing bacteria.

Purification of pGEX-EMBP440 Plasmid:

A culture of bacteria containing pGEX-EMBP440 was grown overnight at 37^{O} C. The plasmid DNA was purified using the PERFECTprep kit.

To check identity and purity of plasmid a restriction digest was carried out. A 35 ul sample of plasmid DNA was added to 5 ul Buffer H (Boeringermanheim, Inc.), 2 ul H_2O and 3 ul ECoR1 at 37^{O} C for 1 hour. Stop buffer was added and the plasmid DNA was run on a 1% agarose gel. An additional 35 ul of uncut plasmid DNA was run on

this gel for comparison purposes and 5ml of HindIII digested lambda phage. DNA Ladder was run as a molecular weight standard.

Purification of GST-EMBP440:

GST-EMBP440 was purified by affinity chromatography as described below. A 250 ul culture of bacteria containing pGEX-EMBP was grown overnight. To this culture 250 ul of IPTG was added and allowed to incubate at 37^{O} C for 2 hours. The bacteria were recovered by centrifugation at 3000 rpm for 2 minutes. The pellet was retained and washed by centrifugation with 50 ml TBS. The washes were repeated 3 times. After the last wash the bacteria were resuspended in 10 ml TBS and placed into a 50 ml test tube. The bacteria were centrifuged at 3000 rpm for 2 minutes and resuspended in 8 ml TBS. The bacteria were lysed by sonication for 1 minute at medium power using point sonication (Branson cell disrupter). Triton X100 was added to the lysate to a final concentration of 1%. The Triton X100 is a detergent that solubilizes inclusion bodies thus making the protein soluble. The lysate was sonicated for an additional 30 seconds at medium power and centrifuged at 3500 rpm for 30 minutes to

remove cell debri from the supernatant. The supernatant was carefully removed and stored at -20^{o} C until further processing.

The supernatant contains all soluble proteins. To purify the protein, the supernatant was loaded and run through a glutathione-agarose affinity resin in a column and collected. The GST-EMBP440 has glutathione-S-transferase activity and binds to the glutathione. The column was washed in 10 bed volumes of 1X TBS for 5 times to wash off any unbound contaminants. The protein was eluted with 5.0 ml of 25mM glutathione and collected in twenty 0.5 ml fractions. A sample of these fractions were run on 12% SDS-PAGE (Laemmli, 1970) to analyze where the protein was located.

To quantify these results and find the concentration of protein, a Bradford protein assay (1976) was performed. This is a dye binding assay using coomassie blue which quantitatively binds to protein. The standard used in the Bradford protein assay was set at 1.35 mg per ml of bovine plasma gamma-globulin. The concentration series used was 1.98 ml of dilute reagent to 20 ul of sample standard in decreasing amounts of 5 ul. Thus, the concentration series started with 20 ul of sample standard and decreased to no sample standard.

Refolding of GST-EMBP440:

The GST-EMBP440 protein was folded into a more native conformation by two methods. The first method involved 0.80 ml of GST-EMBP440 protein added to 0.46 ml ddH_20 to give a total quantity of 1.2 ml. A 9 M urea solution was added at a 2:1 ratio to the sample (therefore 1.2 ml urea to 0.6 ml of sample). This was allowed to set for 1 hour at room temperature and then dialyzed against TBS solution containing TBS and 10% glycerol for 16 hours at 37^{o} C. The dialysis membrane used has a molecular weight cut off of 10,000 daltons (Slide-A-Lyzer, Pierce Chemical Inc.). The urea was dilute 555 fold during dialysis.

In subsequent denaturations of the native GST-EMBP440 protein, 1.0 ml of sample native protein was placed into a dialysis slide bag (Slide-A-Lyzer, Pierce Chemical Inc.) which was allowed to float in a denaturation solution containing 5.422 M guanidine, 44 mM Tris and 22 mM DTT for 16 hours at room temperature. The contents of the dialysis slide were transferred into a microfuge tube and allowed to incubate for 40 minutes at 40^{o} C. The sample was placed back into the slide apparatus. At this point the protocol is

the same in both cases in the renaturation of the GST-EMBP440 protein. The slide apparatus was allowed to float in a room temperature solution of TBS solution as above and was placed at 4° C for 16 hours. The gradual cooling of the solution allows for the renaturation to occur. The slide was placed into fresh TBS solution for an additional 16 hours at 4° C. The contents of the unit in both instances were aliquoted and stored at -20° C.

Purification of Gizzard Myosin:

The purification of gizzard myosin was performed according to the protocol of Persechini and Hartshorne (1983). The method of purification of myosin involves several cycles of high and low ionic strength to polymerize and depolymerize the myosin filaments. The successive assembly and disassembly of the filaments is a process that purifies the myosin, since any proteins that do not associate with the myosin filament remain in the supernatant while the polymerized filaments will pellet.

Four fresh chicken gizzards were obtained. The fatty tissue was trimmed away and the gizzard was cut into two lobes. With a single edged razor, the thin layer of elastic tissue that covers the

outside of the gizzard was removed so as not to be included in the minced tissue. Next, the muscle was cut off of the gizzard and minced. The minced tissue was then weighed. Approximately 20g of minced tissue per gizzard was obtained. The minced gizzards were then homogenized in 3 volumes (3ml/gram) of buffer A (50mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris, 0.2 mM DTT, 3% Triton X100 and 0.2 M ATP) in a 250 ml mixer for 1 minute. It is better to extract with more buffer, as the myosin is associated with the pellet in this treatment. The myofibrils were recovered by centrifugation at 2500 rpm in a GPR rotor for 5 minutes and the supernatant was discarded. The homogenization and sedimentation with buffer A was repeated 3 times. The Triton X100 was washed out of the pellet with 3 cycles of homogenization and sedimentation in buffer B (100 mM KCl, 2 mM EGTA, 10 mM Tris, 0.2 mM DTT and 1.0 mM $MgCl_2$).

The myosin was extracted by suspending the pellet in 2 volumes of extraction buffer (5 mM ATP, 2mM EGTA, 40 mM Imidazole, 0.5 mM DTT and 0.4 M Na_2HPO_4) for 16 hours on ice. This was centrifuged at 12,500 rpm in a Ti 19 rotor for 20 minutes. Myosin was now in the supernatant. The supernatant was filtered through glass wool and the pH adjusted to 7.6. Added to this was 1.0 M $MgCl_2$ which was

added dropwise while the supernatant was stirred on ice to a final concentration of 0.15 M. A 440 ul of ATP was added to the supernatant to give a final concentration of 2.5 mM. The supernatant was centrifuged at 2500 rpm in a GPR rotor for 10 minutes and filtered through glass wool and centrifuged for 16 hours at 19,000 rpm in a Ti 19 rotor. The high speed supernatant was diluted with 10 volumes of cold ddH_2O. The resulting precipitated myosin was collected by centrifugation at 13,000 rpm in a Ti 19 rotor for 15 minutes. There was 24 ml of myosin collected. The pellet was homogenized in 1 volume ddH_2O to which was added to the final concentrations in sequential order 1.5 mM EGTA, 10 mM Na_2HPO_4, 0.2 M $MgCl_2$ and 5 mM ATP in final concentrations. This was allowed to sit on ice for 16 hours to resolubilize the sample and centrifuged at 160,000 g for 3 hours to separate the myosin from any contaminants. A SDS-PAGE gel was run on the resolubilized sample and the extract to verify that myosin was present.

The high speed supernatant myosin was diluted with 10 volumes of cold ddH_2O and allowed to sit for 48 hours. The myosin was collected by centrifugation at 13,000 rpm in a Ti 19 rotor for 15 minutes. The pellet was dissolved in a minimum volume of 0.3 M KCl, 10 mM Tris-HCl pH 7.6 and 0.2

mM DTT for 24 hours. The purity of the myosin was checked by SDS-PAGE and the concentration of myosin was determined by the Lowry assay and by measuring the OD 280. The extinction coefficient of smooth muscle myosin is E= 0.56 $mg/ml^{-1}cm^{-1}$. The myosin was stored in a 30% glycerol solution at -20^{o} C.

Blot Binding to Myosin Assay with Immunodetection:

Blot binding to myosin assays were run according to Walker, Yabkowitz and Burgess (1991). SDS-PAGE on 12% microslab gels and electrophoretic transfer to nitrocellulose blots were performed as described by Yabkowitz and Burgess (1987). Antibodies bound to the nitrocellulose were detected by chemiluminesence. Electrophoresis was performed according to Laemmli (1970) on 0.75 mm thick polyacrylamide gels. There was 10% acrylamide used in the resolving gel. Beta-Mercaptoethanol was present in the SDS-sample buffer to reduce the disulfide bonds.

The first blot binding to myosin assay was run as follows, a SDS-PAGE was run on full length myosin to look at the heavy chains of myosin. The SDS-PAGE was run at a constant current of 0.27 amps with increasing voltage until the dye front ran off. This was done because of the great size of

myosin. The gel was transferred by electrophoretic transfer to a nitrocellulose blot run at 25 volts for 1 hour. The blot was blocked with 50 ml buffer A (7.5 mM KCl, 10 mM PIPES, 0.1 mM EGTA and 0.1 mM ATP), 50 ul 0.1% Tween-20 which blocks unspecific binding, 25 ul 0.1 mM ATP and 30 mg/ml BSA. There was 0.1 mM ATP present in solution at all times to insure proper binding. The blot was allowed to incubate in the blocking solution for 1.5 hours, after which 500 ul of GST-EMBP440 protein and an additional 250 ul of 0.1 mM ATP was added to the blot. The blot was washed with 50 ml buffer A containing 50 ul 0.1 mM ATP 4 times. During the last wash, 100 ul rabbit anti-EMBP440 at a dilution of 1:300 was added and allowed to incubate for 16 hours at $4^{o}C$. The blot was washed with 50 ml buffer A containing 50 ul 0.1 mM ATP for 4 times to remove any excess unbound antibody. To the last wash, 15 ul goat anti rabbit that was peroxidase-labeled was added at a dilution of 1:3000 and allowed to incubate for 1 hour at room temperature. The blot was washed 3 times in 50 ml buffer A containing 50 ul 0.1 mM ATP. The blot was treated for detection with a 1:1 ratio of 10 ml luminol and 10 ml hydrogen peroxide mixture and gently agitated for 5 minutes. The detection was recorded on Fuji Rx film at exposures of 5 seconds,

15 seconds and 30 seconds. The film was developed with Kodak RP X-OMAT developer and fixed with Kodak RP X-OMAT fixer.

The second blot binding to myosin assay was run in a manner similar to the first with some variations. One change is that the SDS-PAGE was run on not only myosin but also the recombinant GST-EMBP440 protein. The blot therefore contained myosin extract, resolubilized myosin and GST-EMBP440 protein. The blot was cut into 3 sections which contained all 3 protein samples in each section. Section 1 was blocked identically to the blot in the first assay. Section 1 was blocked with 20 ml buffer A, 10 ul 0.1 mM ATP, 20 ul 0.1% Tween-20 and 2 ml 20% BSA-H_2O for 16 hours on a rocker. The entire 275 ul recombinant GST-EMBP440 protein sample was added along with an additional 30 ul 0.1 mM ATP and allowed to incubate for 16 hours on a rocker. The blot was washed 4 times with 50 ml buffer A containing 50 ul 0.1 mM ATP for 15 minutes each. To the last wash, 50 ul rabbit anti-EMBP440 at a dilution of 1:1000 was added and allowed to incubate for 1 hour at room temperature. The blot was then washed 4 times with 50 ml buffer A containing 25 ul 0.1 mM ATP for 15 minutes each. To the last wash 15 ul Horseradish peroxidase conjugated goat anti-rabbit was added at a dilution

of 1:3000 and incubated for 1 hour. The blot was washed 3 times with 50 ml buffer A containing 25 ul 0.1 mM ATP for 15 minutes each. The final detection was recorded in the same manner as the first blot.

Sections 2 and 3 of the blot were blocked differently than what was done before. Both sections were blocked with non-fat dry milk (10g non-fat dry milk, 0.4 ml Tween-20 and 200 ml TBS) for 16 hours at 37^{o}C. Section 2 was then removed from the blocking solution and placed into 50 ml TBS containing 50 ul 0.1 mM ATP. To this 50 ul rabbit B anti-myosin at a dilution of 1:1000 was added and allowed to incubate for 1.5 hours at room temperature. The blot was washed 4 times with 50 ml TBS containing 50 ul ATP for 15 minutes each. To the last wash 15 ul Horseradish peroxidase conjugated goat anti-rabbit was added at a dilution of 1:3000 as in the previous blots and allowed to incubate for 1.5 hours at room temperature. The blot was washed 3 times in 50 ml TBS containing 50 ul ATP for 15 minutes each. The final detection was carried out and recorded as described above.

Section 3 of the blot was removed from the blocking solution and placed into 50 ml TBS containing 50 ul 0.1 mM ATP in a manner similar to section 2. To this section 50 ul rabbit

anti-EMBP440 at a dilution of 1:1000 was added and allowed to incubate for 1 hour at room temperature. The blot was then washed 4 times in 50 ml TBS containing 50 ul 0.1 mM ATP for 15 minutes each. To the last wash 15 ul Horseradish peroxidase conjugated goat anti-rabbit at a dilution of 1:3000 was added as and allowed to incubate for 1 hour at room temperature. The blot was washed 3 times in 50 ml TBS containing 50 ul 0.1 mM ATP for 15 minutes each. The final detection was carried out and recorded in the same manner as before.

Binding Assay to Myosin using Affinity Chromatography:

An alternative binding assay utilizing glutathione-agarose affinity chromatography was used to test binding of GST-EMBP440 to non-denatured myosin. Glutathione which specifically binds to the GST-EMBP440 protein was the ligand. Glutathione is covalently attached to an insoluble and porous matrix. Glutathione-S-transferase (GST) is part of the fusion protein and it is this domain of the protein that binds to glutathione. A total of 3 controls were run in separate microfuge tubes containing prebound sepharose beads with glutathione:

a) 50 ul GST-EMBP440 in 100 ul myosin and 25 ul 0.1 mM ATP

b) 50 ul GST-X4 in 100 ul myosin and 25 ul 0.1 mM ATP

c) 100 ul myosin and 25 ul 0.1 mM ATP

Each tube was allowed to incubate with the myosin for 16 hours at 37^{o}C. The samples were washed 3 times in 1X TBS for 15 minutes each. To each control was then added 50 ul of 2X SDS-sample and boiled for 2 minutes prior to being loaded to a 12% SDS-PAGE minigel and run.

IV. Results

Recombinant myosin binding protein (GST-EMBP440) was purified by affinity chromatography. The supernatant containing GST-EMBP440 was run through a glutathione-agarose column and collected. The recombinant protein binds to the glutathione-conjugate agarose since it contains a glutathione-S-transferase domain. The column was then washed with glutathione and TBS, which competes off the bound recombinant GST-EMBP440 protein, and collected in 0.5 ml fractions in 20 labeled microfuge tubes. A sample of these fractions was run on SDS-PAGE gels to analyze the fraction in which recombinant GST-EMBP440 protein was localized (Figure 1 and Figure 2). The majority of the purified recombinant GST-EMBP440 protein was found in fraction #4 (Figure 1, lane 4). Also seen in this gel were proteolytic fragments of the recombinant GST-EMBP440 (Figure 1, lane 4). In subsequent purifications the recombinant GST-EMBP440 protein was identified in a similar manner. The major band in these SDS-PAGE gels is at 56 kilodaltons, which is characteristic of the GST-EMBP440 protein.

The quantity of purified GST-EMBP440 protein was determined by a Bradford protein assay. In a typical purification series, the amount of recombinant GST-EMBP440 protein was similar, being 4.14 mg/ml and 3.820 mg/ml respectively (Figure 3 and Figure 4). These show that the recombinant GST-EMBP440 protein was purified in an ample amount each time.

Myosin was purified by differential precipitation from smooth muscle myosin extracts. An SDS-PAGE gel was run to monitor purification (Figure 5). A molecular weight standard (Figure 5, lane 6) was run with resolubilized myosin (Figure 5, lanes 2 and 4) and myosin extract (Figure 5, lanes 3 and 5). The major bands in lanes 2-5 containing myosin, are at the top of the gel (~200 kDal). This is the characteristic molecular weight of myosin heavy chains. The lower bands in these same lanes suggest either proteolytic fragments of myosin or actin. The band at ~43 kDal is probably muscle actin.

The quantity of myosin was determined by a Lowry assay to be 1.043 mg/ml. This is comparable to the amounts normally purified. The total amount of myosin purified was 22.53 mg.

A myosin blot binding assay was attempted on urea denatured-renatured recombinant GST-EMBP440

protein. The expected results of this assay was to see binding of GST-EMBP440 to myosin. No binding was seen under these conditions (results not shown). One possible explanation for this could be due to the complications in the denaturation and renaturation procedure with the use of urea. Refolding of this protein is known to be achieved by the use of guanidine hydrochloride (Walker,personal communication). Another possible explanation could be that there is no binding site for myosin in recombinant GST-EMBP440 protein. Since the results are inconclusive, a second blot binding assay to myosin was attempted with guanidine hydrochloride used in the denaturation of the recombinant GST-EMBP440 protein. This was performed to determine if the lack of myosin binding was due to complications in the denaturation and renaturation procedure of GST-EMBP440 or if there is no binding site for myosin in GST-EMBP440.

A variation of the first of the myosin blot binding assay was performed using guanidine hydrochloride denatured-renatured recombinant GST-EMBP440 protein. A series of SDS-PAGE gels were run using resolubilized myosin, myosin extract and recombinant GST-EMBP440 protein samples. The inclusion of the recombinant GST-EMBP440 protein

provided an internal control for the antibody detection of GST-EMBP440. Three sets of blots were created this way. Blot binding has high background which is an inherent problem with this type of assay.

The first blot was basically a typical blot binding blot. It was blocked and incubated with renatured recombinant GST-EMBP440 protein. The recombinant GST-EMBP440 protein should bind to the myosin heavy chain bands (~200 kDal) in the blot. The recombinant GST-EMBP440 protein in the blot and the GST-EMBP440 binding to the myosin is detected with the use of primary antibody rabbit anti-EMBP440 which binds to any GST-EMBP440 protein present. The primary antibody was detected with the use of secondary antibody goat anti-rabbit that was peroxidase-labelled. Hydrogen peroxide and luminol were added to the blot so the detection could be seen and exposed to bromide film for 5 seconds. The dark band in the lower left corner of the blot is the lane which contained the recombinant GST-EMBP440 protein (Figure 6, lane 1), indicating that the antibody detection worked. There is no binding of the recombinant GST-EMBP440 protein evident in either of the lanes that contain myosin. This suggests that the recombinant GST-EMBP440 protein does not bind to myosin.

The second blot was used to determine if myosin is indeed present in the blot. The blot was blocked for unspecific binding and the primary antibody rabbit anti-myosin was added. The primary antibody exhibits binding to the lanes containing myosin and not to the lane containing recombinant GST-EMBP440 protein. As with before, the secondary antibody goat anti-rabbit that was peroxidase-labelled was added for detection of the binding of the primary antibody. The final detection was carried out and recorded in the same manner as before. There was no binding evident to myosin in this blot (Figure 7). This suggested that the myosin is absent from the blot. However, the antibody used is ten years old and the binding properties of this antibody are in question.

The last blot was used to determine if the primary antibody showed specific binding to GST-EMBP440. The blot was blocked for unspecific binding and primary antibody rabbit anti-EMBP440 was added to the blot. The primary antibody exhibits binding to the lane that contains the recombinant GST-EMBP440 protein. As was done in the previous blots, the secondary antibody goat anti-rabbit that was peroxidase-labelled was added for the detection of the primary antibody. The final detection was carried out and recorded as

previously on bromide film. A band was recognized in the lane containing the recombinant GST-EMBP440 protein, which suggests that the primary antibody rabbit anti GST-EMBP440 does indeed work (Figure 8).

The blot binding experiment above suggests that the recombinant GST-EMBP440 protein does not bind to smooth muscle myosin. An alternative myosin binding assay was thus devised to confirm the above results. A glutathione affinity precipitation assay was used to determine if myosin binding occurred with recombinant GST-EMBP440 protein. As mentioned before, the advantages of this technique is its ability to exploit the ability of glutathione-S-transferase to bind glutathione. In this case, recombinant GST-EMBP440 protein has a glutathione-S-transferase domain. Three controls were run. Glutathione-agarose was incubated in separate microfuge tubes with GST-EMBP440, GST-X4, and glutathione-agarose alone as a negative control. Both the GST-EMBP440 and GST-X4 exhibit the ability to bind to glutathione because these contain a glutathione-S-transferase domain. Myosin was incubated with all three samples to determine if binding would occur. After washing the agarose beads, the samples were run on SDS-PAGE gel to detect binding to myosin. The results demonstrate

that myosin binds to the recombinant GST-EMBP440 fusion protein glutathione-agarose precipitate (Figure 9, lanes 3 through 6). This is evident by the presence of myosin heavy chains (~200 kDal) as well as GST-EMBP440 (~56 kDal). This finding suggests that myosin binds to GST-EMBP440 (Figure 9, lanes 3 through 6), since myosin by itself (Figure 9, lane 10) does not bind to glutathione agarose beads and there was only residual binding of myosin to GST-X4 (Figure 9, lanes 8 and 9).

V. Discussion

Myosin is a molecular protein of great importance in many cell types: for instance, it comprises 50% of the total protein in skeletal muscle (Harrington and Rogers, 1984) and contributes to movements, ranging from contraction to cytokinesis, in non muscle cells (Kiehart, 1990). Thus, the discovery and development of proteins that can regulate myosin is of great use and interest of science.

The binding assay to myosin using affinity chromatography suggests that the recombinant GST-EMBP440 fusion protein binds to native myosin. This finding is consistent with it being the native myosin binding protein in sea urchin eggs (Yabkowitz and Burgess, 1987) and with other studies indicating that GST-EMBP440 is a myosin light chain kinase (Walker, unpublished data).

The differences seen in myosin binding in the two binding assays may be due to structural differences between myosin in solution and myosin on the nitrocellulose blot. This structural difference may involve the disruption of the heavy chain head-rod junction of myosin since this is where the binding occurs on the native 53K protein

(Walker, Yabkowitz, and Burgess, 1987). Myosin in the blot binding assay is denatured during the process of running SDS-PAGE. With glutathione affinity precipitation, the myosin is not denatured but remains in a native conformation.

The myosin binding activity indicated by the glutathione-agarose affinity precipitation assay, along with the knowledge that this protein shows myosin light chain kinase activity (Walker, unpublished data), suggests an important role of this protein in myosin regulation. Myosin structure is affected by phosphorylation, causing it to change shape from a 10S to 6S form depending on the state of myosin phosphorylation. The myosin binding activity of recombinant GST-EMBP440 is also consistent with the observed association of native 53K protein with myosin complexes (Walker, Yabkowitz and Burgess, 1991). These are believed to be large assemblies of myosin bipolar filaments.

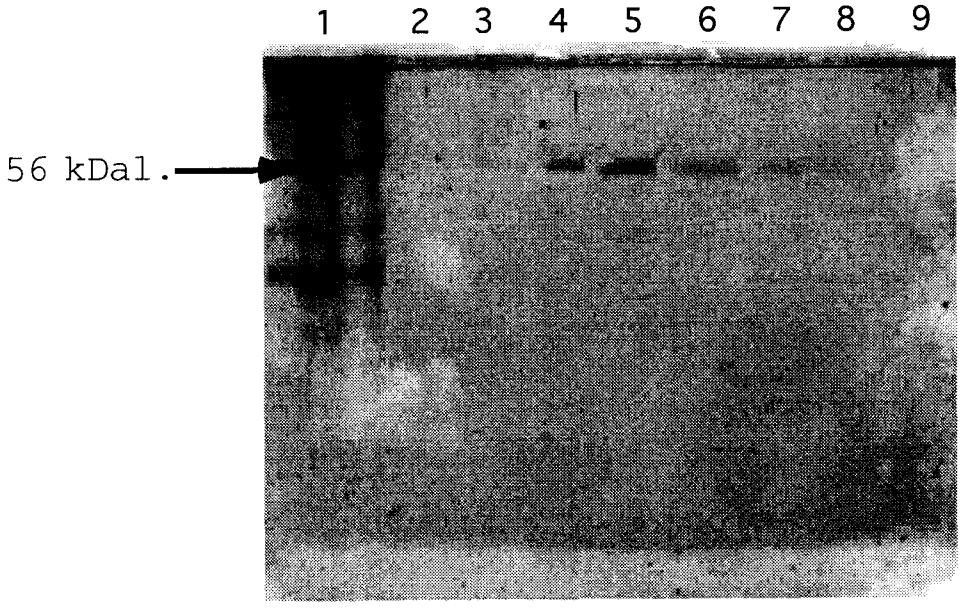
A future experiment will be to examine the effects of the recombinant GST-EMBP440 fusion protein on myosin's solubility. When the native 53K protein was depleted from cell extracts, a majority of the myosin precipitates under low ionic strength conditions (Yabkowitz and Burgess, 1987). Native 53K protein restores the low ionic strength solubility of myosin in fresh egg extract.

(Yabkowitz and Burgess, 1987). Recombinant GST-EMBP440 protein can be reconstituted with myosin to test whether it to can restore low ionic strength solubility of myosin.

Another possible experiment is one that examines the level of nucleoside triphosphate dependence of the recombinant GST-EMBP440 protein on myosin binding. While the binding of native 53K protein to myosin appears to be dependent upon nucleoside triphosphates (Yabkowitz and Burgess, 1987), the level of this dependence remains unclear. Future experiments may examine nucleoside triphosphate dependence by the recombinant GST-EMBP440 fusion protein on myosin binding. This may be done by varying the types and concentrations of nucleoside triphosphates which are added to GST-EMBP440 protein and myosin solutions to determine what effects this may have on myosin binding by GST-EMBP440 protein.

The native 53K protein is distributed much the same as myosin in embryonic mouth and gut smooth muscle (Walker, unpublished data). The native 53K protein may have structural properties on myosin in those regions (Walker, unpublished data). The native 53K protein may also play a role in localizing myosin to the contractile ring during cytokinesis. These questions remain to be answered

concerning native 53K protein and its effects on myosin.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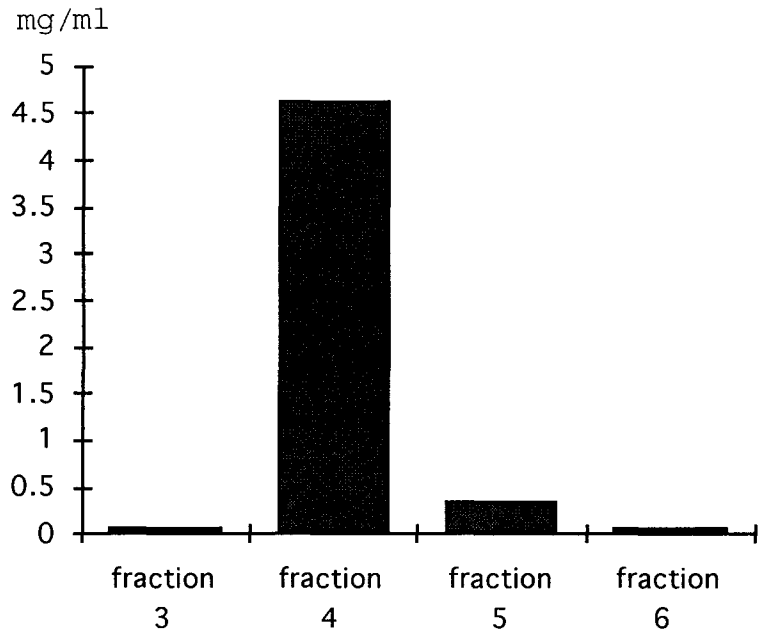
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0
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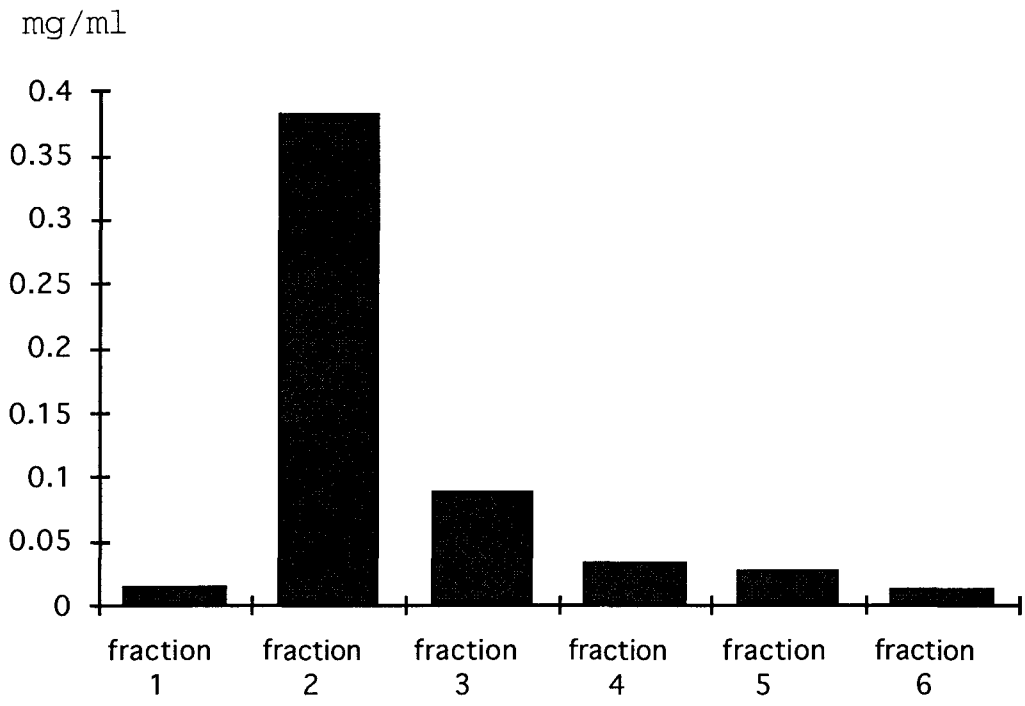
fraction 4
fraction 5
fraction 6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0
fraction 1
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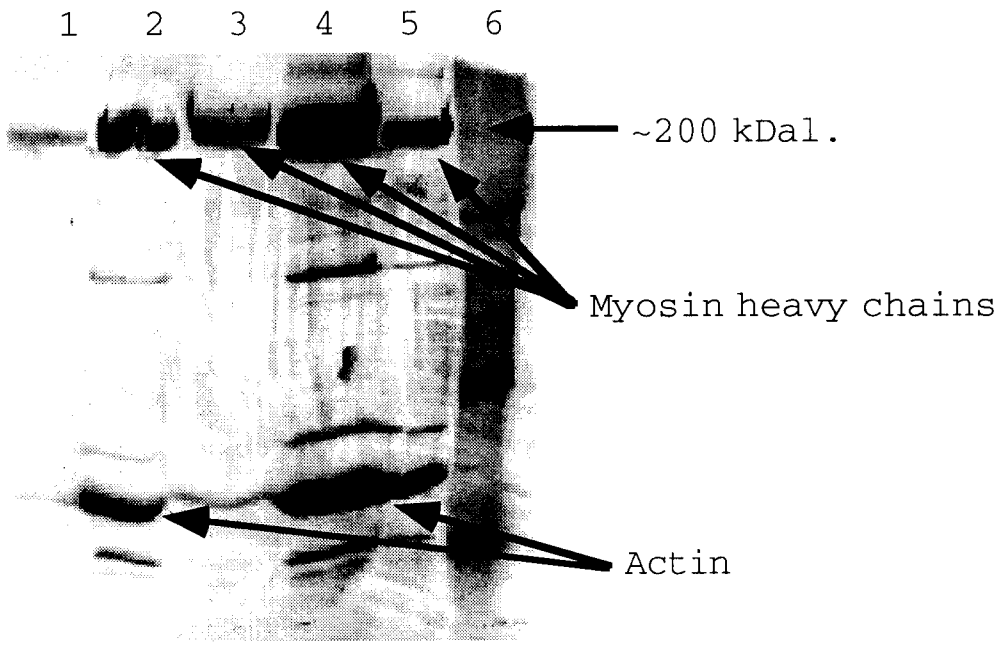
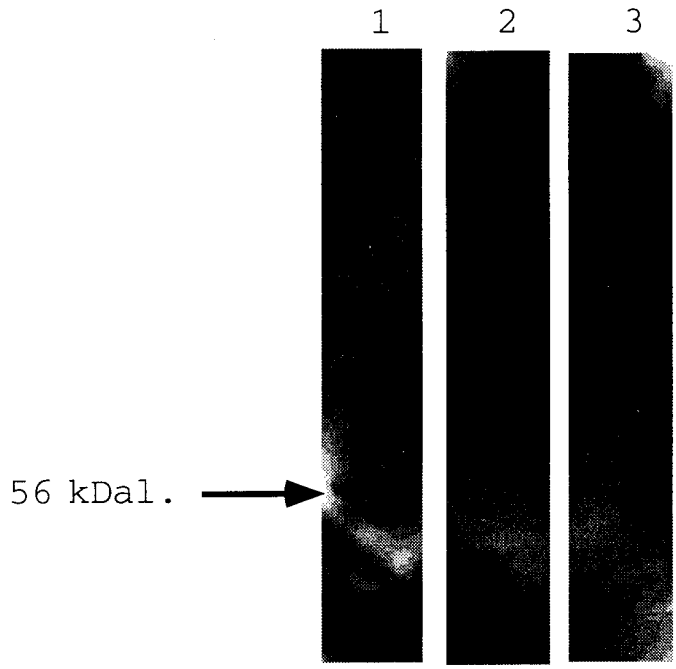
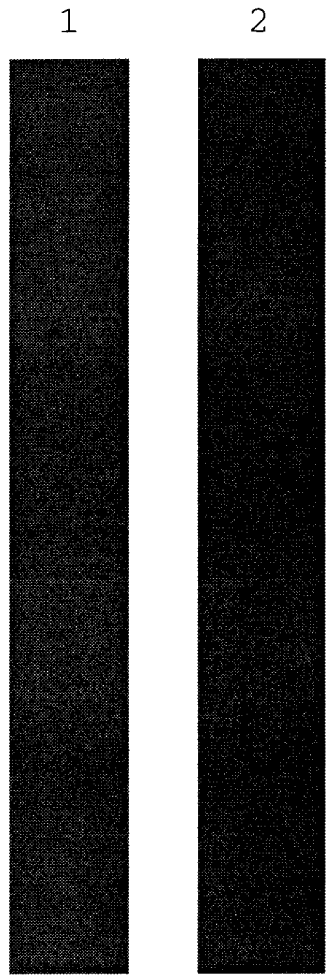
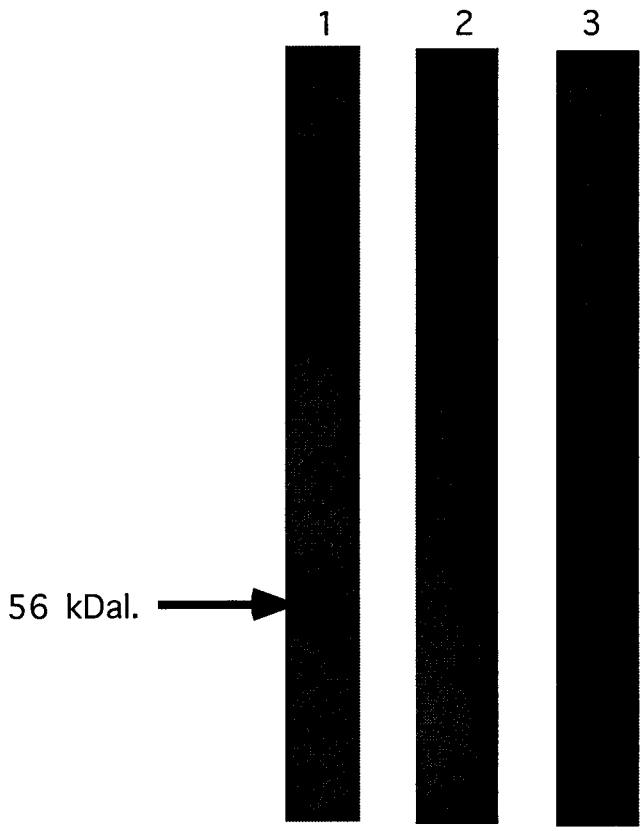
fraction 4
fraction 5
fraction 6


1
2
3
4
5
6
~200 kDal.
Myosin heavy chains
Actin


1
2
3
56 kDal.


1
2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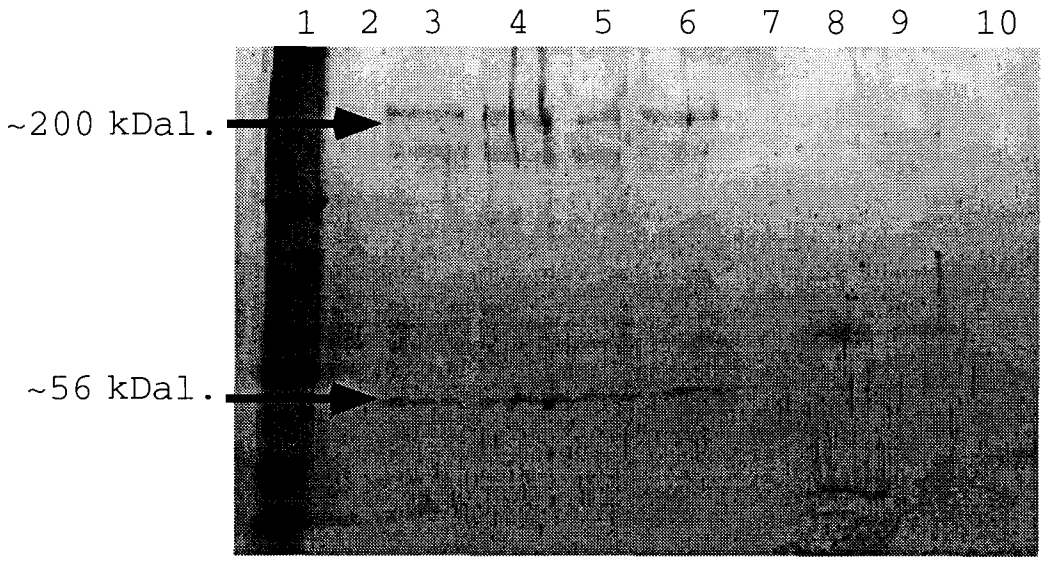
1 2 3 4 5 6 7 8 9 10
~200 kDal.
~56 kDal.

VI. References

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