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Microsequencing of Myosins for PCR Primer Design

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1. Introduction

1.1. Background

Their large size and their relative resistance to proteolytic cleavage (1) make myosins particularly difficult substrates for the acquisition of their peptide sequences by standard protocols. For this reason, instead of identifying myosins first according to their biochemical activity and then obtaining their sequences, PCR and other DNA-based techniques exploiting the highly conserved sequences in the amino end, the “head” domain, have been used to find new myosins (2–5). However, such identification of myosins by sequence leaves open the question of their function. If peptide sequence could be obtained from myosin proteins whose biochemical behavior was known, then the gap between function and sequence could be bridged. We describe here a method that enabled us to acquire peptide sequences of semi-purified myosins (6).

1.2. Rationale for the Strategy

There are several difficulties that must be overcome to obtain peptide sequences from myosins. First, a sufficient amount of protein that is reasonably pure must be obtained. For muscle and non-muscle myosin IIs this is rarely a problem, although it can pose problems if the organism or the tissue to be studied is small, rare or otherwise difficult to obtain in large quantities—as is the case for axoplasm from the squid giant axon (6–10) or the organism *Tetrahymena* (2). This chapter will not address problems of abundance since they pertain to individual applications.

Once sufficient amounts (micrograms) of a myosin have been obtained, the protein must be proteolytically cleaved into peptide fragments. These fragments must then be purified to homogeneity for Edman digestion to produce readable sequence. Myosin is relatively protease resistant. The coilcoil region in the tail and the tight packing of the amino head

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²To increase the yield of protein from a protein purification preparation, we decreased the number of solution purification steps and used gel electrophoresis as the final step at an earlier stage in the purification protocol. This strategy also helps eliminate contamination of the soluble purified myosin with proteins of different molecular weights, although it allows higher levels of contamination with proteins of similar molecular weight. Many of the more abundant proteins in most systems have been cloned and sequenced, and such contaminants can usually be eliminated by databank identification as a non-myosin. By digesting in the gel and eluting the resultant peptide fragments, we were able to overcome the problems encountered by poor transfer of intact myosins out of the gel onto PDVF membranes.

³For in-gel digestion: Protein is electrophoretically purified through a 6% polyacrylamide-SDS gel to facilitate separation of high molecular weight species and to increase the yield of peptides eluting from the gel after digestion. We use 0.8% *bis*-acrylamide, not 1% as recommended in Maniatis. Sometimes better separation can be obtained with 0.6% *bis*. The gel is stained with Commassie, destained, and mounted in water on an acid-cleaned glass plate. A razor, cleaned with ethanol and rinsed in bleach, is used to excise the band of interest. Gel bands are stored in distilled water and can be stored at –70°C. A high protein to acrylamide ratio results in a better yield from the digest. Twenty gel slices with 0.5–10 µg of protein each are digested in 140 µL of DB with 1.5 µg of endoproteinase Lys-C overnight at 37°C. The solution is removed from the tube and another 150 µL of DB added and allowed to soak for 1 h at room temperature. This second “wash” is pooled with the digest and 50 µL of 6M guanidinium added and the sample vortexed to precipitate the SDS. The precipitate is removed by passage through the Millex filter and then processed by HPLC as described above.

domain prevent cleavage at all but the neck region that links the two domains. Chymotrypsin, trypsin and pronase all produce cleavage in the neck and leave the rest of the molecule intact even under conditions when other proteins would be reduced to peptide fragments. Since tight packing of these domains in myosin is likely responsible for this protease resistance, we unwind the myosin before proteolysis with sodium dodecyl sulfate (SDS), although other chaotropic agents may work as well. While low levels of SDS unwind myosin, the proteolytic enzyme endolys C, a serine protease, remains active in SDS. This strategy results in sensitivity to proteolysis throughout the myosin molecule.

After proteolytic cleavage, the resultant peptide fragments must be separated. For a large molecule this is particularly difficult as complete digestion results in a very large number of small peptides, often with very similar properties making them difficult to separate. Hence, chromatographic separation must be performed in two steps in order to obtain adequately pure peptides. Several factors including inadequate amount of starting material, incomplete digestion of proteins, or loss of peptides during the chromatographic separations, can lead to insufficient quantities of each purified peptide for the final sequencing reaction.

The following protocol was first developed using squid muscle myosin as starting material. At the time, that myosin had not been purified, and its sequence had not been obtained. The sequence of squid muscle myosin II was ultimately obtained by a combination of two approaches: (1) PCR sequencing using primers to hypothetically homologous domains from scallop muscle myosin, a myosin from a related species, and (2) PCR sequencing using degenerate PCR primers based on the peptide sequences we obtained from the purified squid protein (11,12). After devising a strategy applicable to muscle myosins, we then applied this strategy to the identification of a squid brain myosin not so easily obtained in large amounts (6,10,13,14). Others have since applied our strategy to obtain sequences from other squid optic lobe myosins (15). This strategy is therefore likely to be of use for most myosins even those that are significantly less abundant than muscle myosin. This method can be applied to purified myosin in solution or semi-pure myosin excised from a Coomassie-stained SDS-PAGE gel. The strategy as we applied it was a compilation and modification of previously reported methods (16–18).

2. Materials

2.1. Reagents and Glassware

1. Endoproteinase Lys-C (Wako Chemicals, Richmond, VA).
2. Guanadinium hydrochloride (Sigma Chemicals, St. Louis, MO).
3. Biospin-30 chromatography column (BioRad, Hercules, CA).
4. Millex HV filter (Millipore, Bedford, MA).
5. Vydac 218TP52 column (Separations Industries, Metuchen, NJ).
6. YMC ODS AQ column (YMC, Willmington, NC).
7. Briobrene-treated glass fiber filter (Applied Biosystems, Foster City, CA).

2.2. Buffers

1. Myosin storage buffer (MS): 0.5M KCl, 3 mM Na₃N, 2 mM MgCl₂, 1 mM DTT, 2 mM EGTA, 4 mM NaHCO₃ (pH 7.0), 50% glycerol.
2. NED: 0.1 mM NaHCO₃, 0.1 mM EGTA, 0.1 mM DTT.
3. Digestion buffer (DB): 0.1 % SDS, 100 mM NH₄HCO₃.

4. Solvent A: 0.1% trifluoroacetic acid in water.
5. Solvent B: 0.1% trifluoroacetic acid in acetonitrile.

2.3. Equipment

1. Beckman System Gold HPLC equipped with an autosampler (model 507), diode array detector (model 168), and programmable solvent module (model 126).
2. Applied Biosystems pulsed-liquid protein sequencer (model 477A) equipped with PTH analyzer (model 120A).
3. Applied Biosystems data analysis system (model 610A).

3. Methods

3.1. Proteolytic Digestion of Myosins (see Notes 1–4)

1. Precipitate purified myosin out of buffer (MS) by adding a tenfold volume of NED to 40 µg of the myosin suspension.
2. Incubate for 1–4 h on ice.
3. Collect the precipitate by centrifugation at 16,000*g* in a microfuge for 30 min (*see* Note 1).
4. While the myosin is precipitating, equilibrate the BioSpin 30 column. First remove the packing buffer by centrifuging in the 2 mL tube (comes with the kit from BioRad) for 1 min at 1,000*g* or until the entire amount has passed through the column. Discard the buffer. Next add 100 µL of DB to the top of the column and centrifuge again. It is best to use a swinging bucket rotor, but you can also do this with a benchtop microfuge. After the DB has passed through the column, remove it, and let the tube sit on the bench until loading.
5. Resuspend the myosin precipitate in 80 µL of DB. Heat to boiling (95–100°C) for 5 min.
6. Apply 80 µL of denatured protein solution to the DB-equilibrated BioSpin 30 column and centrifuge the tube for 4 min at 1000*g*. This removes light chains that are released during the denaturation process, and any other contaminating proteins smaller than 40 kDa which are retained in the column. Dispose of the column, and use the solution that passed through it for subsequent steps.
7. Add to the column eluate 6 µL of DB containing 1.5 µg of endoproteinase Lys C. Put samples in 37°C water bath and allow to digest overnight (16 h).
8. Add 150 µL of DB and 150 µL of 1*M* guanidinium hydrochloride to the digests. The SDS will precipitate. Clarify the protein suspension by centrifugation for 15 min in a microfuge at full speed, room temperature.
9. Pass the supernatant through a Millex HV filter unit to remove any residual SDS precipitates.

¹Myosins that do not precipitate in NED can also be pelleted with addition of 1/10 volume of trichloroacetic acid for 15 min on ice. The resultant precipitate is collected by centrifugation in a microfuge at 16,000*g* for 15 min at 4°C.

⁴To improve proteolytic digestion, SDS is used to open proteolytic sites in the myosin, particularly in the head and coil-coiled domains in the tail. Alkylation and reduction do not improve digestion for these particular proteins. However, other myosins or other high molecular weight proteins may expose protease sensitive sites if they are alkylated and reduced. Alkylation/reduction can be achieved by addition of an equal volume of 45 mM dithiothreitol (DTT) to the sample and incubating for 15 min at 40°C before digestion.

10. While the SDS is precipitating, prepare HPLC Vydac 218TP52 column by equilibrating in 1.6% Solvent B in Solvent A.

3.2. Isolation of Peptides

1. Load the entire 300 μ L sample into the loop of the input line to the HPLC column.
2. Elute column at 0.25 mL/min with a three step gradient. We used a binary solvent delivery system. Step 1: 1.6–29.6% Solvent B in Solvent A (0–63 min, 15.75 mL). Step 2: 29.6–60% Solvent B in Solvent A (64–95 min, 7.75 mL). **Step 3:** 60–80% Solvent B in Solvent A (95–105 min, 2.5 mL).
3. After the final elution, wash the column for 12 min in 80% Solvent B in Solvent A and then re-equilibrate in 1.6% Solvent B in Solvent A.
4. Monitor protein concentration at 215 and 280 nm wavelength. Collect fractions at 30 s intervals and store them at -70°C . Select one fraction from each of the highest, narrow peaks that eluted at least several minutes apart. The fractions selected should be as symmetric (up slope similar to down slope) as possible. Such symmetric, sharp, high peaks are most likely to contain one or only a few peptides species.
5. Pool the separated peaks for loading on the second column. This eliminates the peptides eluting between these higher peaks. By loading these fractions onto a second, different C18 (18 carbon-chain) reverse phase column peptides whose elution is overlapping through the first chromatographic step, can be separated into the empty intervening elution. Even though the second column is also a C18 column, it behaves differently enough to separate the same set of peptides that were not separated on the first column.
6. Remove the acetonitrile by evaporation in a Savant Speed Vac (centrifuge for 1 h/ 100 μ L of sample). While the sample is in Speed Vac, equilibrate the second column, YMC ODS AQ column, in 1.6% Solvent B in Solvent A.
7. Load the sample (max 500 μ L) into the loop of the HPLC and elute with the same series of step gradients as for the first column, at the same rate (0.25 mL/min) at 35°C .
8. Monitor protein concentrations as before. Collect 0.125 mL fractions at 30 s intervals and store them at -70°C (*see* Note 5).

3.3. Peptide Sequencing

1. Select a fraction from a narrow high peak and thaw rapidly. Apply 30 μ L onto a Briobrene-treated glass fiber filter loaded into the drying arm of the 477a sequencer.

⁵To improve the separation of co-eluting peptides, two C18 hydrophobic columns can be used in sequence. Although these columns retain and elute peptides under similar conditions, they are made by different manufacturers. The YMC ODS AQ column differs enough from the 218TP52 column that it successfully separates peptides that co-elute on the 218TP52 column. By removing the smaller peaks between the major peaks, a blank in elution space is provided such that the newly separated peptides do not co-elute with some other species. Even with this strategy, some co-elution of peptides is still encountered. Since we obtained so many peaks, we were able to obtain many peptide sequences of clean peaks and could ignore those with overlapping amino acid sequences (6). However, if there are not many peaks, one approach might be to take smaller fractions that elute at greater distance from each other. If no peptides are separated by this sequence of columns, a C18 column from a different manufacturer could be tried, or a C16 column be substituted. The advantage of using two similar columns is that the same solvent conditions that are known to work in the first column can be reapplied in the second and the peptides that were initially isolated will be recovered at high efficiency.

2. Lower the drying arm. When the 30 μ L is dried down onto the filter, load another 30 μ L and dry and so on until the entire sample has been dried down onto the fiber filter.
3. Load the filter into the cartridge of the sequencer and then activate the machine.
4. Collect amino acid profiles amino acid profiles and analyze on the data analysis machine.

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