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MONOCLONAL ANTIBODIES AS A TOOL FOR STUDYING THE PROTEIN-STRUCTURE RELATIONSHIP IN FISH MYOSIN

ANTICUERPOS MONOCLONALES COMO HERRAMIENTA PARA EL ESTUDIO DE LA RELACIÓN PROTEÍNA-ESTRUCTURA EN MIOSINA DE PEZ

ANTICORPOS MONOCLONAIOS COMO FERRAMENTA PARA O ESTUDO DA RELACIÓN PROTEÍNA-ESTRUCTURA EN MIOSINA DE PEIXE

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Abstract

Fish muscle protein presents unique properties such as reduced frozen storage stability and setting phenomena. Both two are associated with myosin interactions and are highly dependent on fish specie. Although protein functional properties are highly correlated with amino acid sequence and with distinct structural domains, it is very difficult to determine this relationship for fish myosin, due to its high molecular weight and the great varieties of fishes. The present study deals with the feasibility for studying such relationship employing available isolated monoclonal antibodies whose epitopes has been well-characterized on chicken myosin heavy chain. This alternative opens a new way to be explored.

Keywords: immunoassay, monoclonal antibodies, myosin, fish.

Resumen

La proteína de los peces presenta propiedades únicas, tales como la reducida estabilidad al almacenaje en congelación y el fenómeno de asentamiento. Ambas están asociadas con interacciones de la miosina y son altamente dependientes de la especie de pez. Aunque las propiedades funcionales de las proteínas están muy relacionadas con la secuencia de aminoácidos y con distintos dominios estructurales, es muy difícil determinar su relación para la miosina de la proteína de los peces, debido a su alto peso molecular y la gran variedad de peces. El presente estudio trata sobre la viabilidad de estudiar esa relación empleando anticuerpos monoclonales aislados y disponibles comercialmente, cuyos epitopes han sido caracterizados para la cadena pesada de miosina de pollo. Esta alternativa abre una nueva vía para ser explorada.

Palabras clave: immunoensayo, anticuerpos monoclonales, miosina, peces.

Resumo

A proteína dos peixes apresenta propriedades únicas, tal como a reduzida estabilidade ao armazenamento e ó fenómeno de asentamento. Ambas estão ligadas a interacções da miosina e são moi dependentes da especie de peixe. Ainda que as propriedades funcionais das proteínas esteñam moi relacionadas ca secuencia de aminoácidos e cos dominios estruturais, é moi difícil determinar a su relación ca miosina da proteína dos peixes, debido ó seu alto peso molecular e a gran variedade de peixes. O presente estudo trata de evaluar a viabilidade de estudar esa relación empregando anticorpos monoclonais aíllados e dispoñíbels comercialmente, cuns epitopes que foron caracterizados para a miosina do polo. Esta alternativa abre unha nova vía para ser explorada.

Palavras clave: immunoensaio, anticorpos monoclonais, miosina, peixes.
INTRODUCTION

Myofibrillar proteins are responsible for the functional properties such as gelling, water holding capacity and binding in animal meat products. Despite the similar role on functionality, fish muscle proteins exhibit unique functional properties. The principals are reduced frozen storage stability and setting phenomena (Toyoda et al., 1992; MacDonald and Lanier, 1991). The latter is the ability of fish myofibrillar proteins to gel between 5 and 43 °C, as a function of incubation time, after solubilization with 2-3% of salt (Toyoda et al., 1992). Recently it has been proposed that such differences could be attributed to the fact that fish are cold-blood animals while poultry and mammals are warm-blood (Toyoda et al., 1992). Moreover, some fish species are more susceptible to frozen instability than others. Setting phenomena is correlated with different rates and optimal temperatures depending on the species (Kamath et al., 1992). The instability during frozen storage has been associated with a lose of stability of fish myosin (the major contractile protein of skeletal muscle), and is dependent on habitat temperature, with myosin from cold-water species being less stable than myosin from warm-water species (Davies et al., 1994). On the other hand, the unique setting phenomena observed in fish myofibrillar proteins is also associated with myosin interactions (Nowas et al., 1992). Although myosin has been implicated in both properties, it remains unclear if there is a structure-function correlation between myosin from different fish species and functional properties.

Immonoassays employing specific monoclonal antibodies have been used to:

1) determine evolutionary relationships among myosin from different species (Miller et al., 1989),
2) determine solubility properties (Wick et al., 1996),
3) identify different chicken skeletal myosin isoforms present in embryonic, neonatal, adult fast, slow and gizzard muscle (Miller et al., 1989).

There are different myosin isoforms in vertebrates that are recognized by specific monoclonal antibodies, indicating differences in protein primary structure. Chicken fast myosin isoforms, present mainly in white muscle, and slow myosin isoforms present in red muscle, differ in ATPase activity (Moore et al., 1992), thermal properties (Xiong et al., 1987), gelling capacity (Xiong, 1994), amino acid sequence and epitopes for specific monoclonal antibodies (Moore et al., 1993; Bandman, 1992).

Vertebrate myosins present similar structure and functional properties. However some functional properties vary among species and the origins being unclear. It is possible that amino acid sequence more than amino acid composition is the answer. The amino acid sequence dictates the three-dimensional structure of a protein and therefore its thermodynamic stability, charge distribution, hydrophilic and hydrophobic characteristics, and has been reported to be important to the structure-function relationship (Damodaran, 1994). However, fish myosins have not yet being studied to determine their primary structure. This may be due to the number of fish species, the high molecular weight of this protein and the fact that the determination of amino acid sequence is not an easy task. Therefore, immunochemistry, using monoclonal antibodies has been an alternative to demonstrate differences among myosin heavy chains (MYHCs) isoforms in the same muscle and for comparison among different MYHC isoforms in distinct species.

Several studies have shown that monoclonal antibodies that react with chicken myosins also reacted with myosins from other animals. The epitope for the 5C3 antibody has been reported to be present in rabbit psoas muscle which contains fast skeletal MYHC isoforms (Winkelmann and Lowey, 1986), and F59 reacts with the S-1 from different vertebrate MYHC isoforms such as, chicken, quail, rat, rabbit, turtle, newt, frog, goldfish, electric ray and shark, but did not react with nematode, slime mold and amphioxus the invertebrate MYHCs (Miller et al., 1989).

On the other hand the 10F12 and 12C5 chicken myosin antibodies did not react with rabbit myosin from psoas muscle (Winkel and Lowey, 1986). Other antibodies, not used in this research, like F18, F27, F30 and F49, whose epitopes are present on chicken myosin S-1, reacted with other vertebrates, such as quail, rat and rabbit (Miller et al., 1989).

The objective of this work was to determine if fish skeletal muscle myosin from Tilapia nilotica shares some of the epitopes observed on chicken skeletal muscle myosins. The seven specific monoclonal antibodies employed on this work, were selected because their epitopes have been well ubicated on the chicken myosin (Table 1), and so, if they are present in the same fish myosin regions, they could be an excellent tool to study differences on functional properties among fish species, associated with differences on protein structure.

MATERIAL AND METHODS

Myosin extraction and purification

Alive Tilapia nilotica fishes were obtained from a Querétaro, México dam. Myosin was extracted from 30 g of sample obtained from several fishes, according to Martone et al. (1986). The extracted myosin was solubilized in 0.6 M KCl, 20 mM Tris-HCl (pH 7.0) and stored for 7 days at 4 °C because to transportation requirements. Subsequently, samples were dialyzed against 80 mM sodium pyrophosphate, 2 mM magnesium chloride, 2 mM EGTA, pH 9.5 buffer, after which glycerol was added to make an 1:1 glycerol-buffer solution and stored for 3 weeks at -20 °C. Myosin was further purified by ion exchange chromatography as described by Richards et al. (1967). The entire process was performed at 4 °C.
Table 1. Reported specificity and epitopes localization for chicken skeletal muscle myosin antibodies.

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>SPECIFICITY</th>
<th>FRACTION</th>
<th>LOCALIZATION (RESIDUES)</th>
<th>DISTANCE (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5C3</td>
<td>Adult MYHC fast isoforms [1, 2]</td>
<td>LMM</td>
<td>- - -</td>
<td>129.29</td>
</tr>
<tr>
<td>NA4</td>
<td>All sarcomeric MYHC isoforms [3-4]</td>
<td>LMM</td>
<td>994-1004</td>
<td>125.69</td>
</tr>
<tr>
<td>EB165</td>
<td>Embryonic and adult MYHC fast isoforms [3–4]</td>
<td>LMM</td>
<td>804-810</td>
<td>55.77</td>
</tr>
<tr>
<td>AB8</td>
<td>Adult MYHC isoforms [3-4]</td>
<td>LMM</td>
<td>428-470</td>
<td>46.12</td>
</tr>
<tr>
<td>10F12</td>
<td>Adult MYHC fast isoforms [5]</td>
<td>S-2</td>
<td>- - -</td>
<td>- -</td>
</tr>
<tr>
<td>12C5</td>
<td>Adult and embryonic MYHC fast isoforms [2, 5–6]</td>
<td>S-1</td>
<td>29-60</td>
<td>14.00</td>
</tr>
<tr>
<td>F59</td>
<td>All sarcomeric fast MYHC isoforms [1]</td>
<td>S-1</td>
<td>211-231</td>
<td>- -</td>
</tr>
</tbody>
</table>

Localization: From NH2 terminus to COOH terminus.
Distance: From the myosin head/rod junction.
-- Data not reported in reference

Figure 1. Illustration of the sites where monoclonal antibodies bind to chicken skeletal myosin. Distances showed are according with the localization of residues indicated on Table 1, except for 5C3 and 10F12. (Adapted from Wick et al., 1996).

Purity of the selected fractions was confirmed by SDS-PAGE (7% T). Fractions were concentrated by dialysis against glycerol.

Electrophoresis and Immunoblotting

Protein samples were prepared for electrophoresis by mixing protein solution (0.2 to 0.5 mg/mL) with 5X sample buffer and boiling the sample for 3 min. SDS-PAGE was performed according to Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose paper as described by Bandman and Zdanis (1988). The transfers were incubated with monoclonal antibodies at various dilutions (i.e. 1:500 or 1:1000) for 60 min at room temperature, in phosphate buffered saline pH 7.4, 5% nonfat dry milk (BBSM).

NA4, 5C3, EB165 and AB8 antibodies were raised at the Department of Food Science and Technology, University of California at Davis. F59 was supplied by Frank E. Stockdale from the Department of Medicine, Stanford University School of Medicine, Stanford, Ca. 12C5 and 10F12 were supplied by Susan Lowey from Department of Biochemistry, Rosenstiel Basic Medical Science Institute, Miami, Fl.
Preparation of S1, light meromyosin (LMM) and rod fraction

S1 was prepared according to Margossian and Lowey (1982). Purity was determined by SDS-PAGE. The S1 fraction was concentrated by dialysis in glycerol. The final solution of S1 was obtained by dialysis against 0.6 M KCl, 0.15 M potassium phosphate (K₂HPO₄), at 4 °C overnight. Despite results reported with rabbit, in fish myosin EDTA did not protect the neck region and the described proceeding resulted in the production of S-1, LMM and rod simultaneously. After removal of S1, the insoluble precipitate (containing myosin, rod and LMM) was dispersed in 0.6 M KCl, 0.15 M potassium phosphate, pH 7.0, and rod and LMM fractions were isolated according to Margossian and Lowey (1982). A 0.6M KCl, 0.15 M potassium phosphate buffer, pH 9.5, 0.001 M DTT was employed instead of 0.03 M KCl, 0.01 M potassium phosphate buffer, pH 7.0 recommended. Under these conditions, much of the LMM and rod were solubilized, whereas the denatured myosin, which remained insoluble, along with insoluble rod and LMM was removed by centrifugation. Purity was determined by SDS-PAGE. Both fractions were pooled and concentrated by dialysis against glycerol. The final solution of rod and LMM was obtained by dialysis against 0.15 M potassium phosphate (K₂HPO₄), 0.6 M KCl at 4 °C overnight.

RESULTS AND DISCUSSION

Purified fish myosin rod, LMM and S-1 preparations were employed to determine the location of the epitope for each monoclonal antibodies. The specificity of the monoclonal antibodies employed in this study are indicated in Table 1. Their epitopes are found in different regions of LMM (S-2) and S-1 (Fig. 1). Monoclonal antibodies, whose epitopes have previously been reported to reside in LMM domain in chicken myosin, reacted with both LMM and rod fractions in fish myosin (see Fig. 2). Antibodies whose epitopes were located in the S-2 domain reacted only with the rod in fish myosin (Fig. 3).

Five of the seven chicken myosin monoclonal antibodies reacted with the fish myosin: 5C3, NA4, EB165, 10F12 and F59 (Fig. 2 and 3). The AB8 and 12C5 antibodies did not react with fish myosin.

5C3, NA4 and EB165 monoclonal antibodies, reacted with both fish myosin rod and LMM, but not with S-1 (Fig. 2). These results indicate that their epitope must be on the LMM fraction of fish myosin, which is similar to chicken myosin. The NA4 gave a special increase on sensitivity, showing a greater background, detecting fractions of rod in purified S-1 fractions, which were not detected by electrophoresis. The 10F12 antibody stained only the band corresponding to the myosin rod fraction (Fig. 3A). The F59 monoclonal antibody reacted with the fish myosin S-1 preparation (Fig. 3B), but did not react either with rod or LMM indicating that the F59 epitope is located on the head domain of fish myosin. These results indicate that the five monoclonal antibodies, which reacted with fish skeletal muscle myosin, did so with the same myosin regions reported for chicken skeletal myosin.

Because monoclonal antibodies only react with the identical primary sequence, there is likely that epitopes are in the same location (Fig. 1). The discrepancies in the location of the antibodies are a common occurrence (Wick et al., 1996). The position of the AB8 antibody visualized on the rod (Table 1) was consistent with the position of the antibody epitope as determined by deletion set mapping (Moore et al., 1992). The position of EB165 antibody visualized by electron microscopy was closer to the N-terminus of the LMM than predicted by deletion set mapping. However, in the rod, the antibody reacted with several regions of the LMM.
similar discrepancies with antibodies that bound near the C-terminus of the myosin rod (Rimm et al., 1990). On Figure 1, the epitopes were ubicated according with their position determined by deletion set mapping (Table 1).

Our results demonstrate that five of the seven monoclonal antibodies studied, reacted with the same domains in chicken and fish MYHC. 5C3, NA4 and EB165 epitopes were located in the LMM of fish MYHC, likewise, F59 reacted with S-1 and 10F12 only reacted with the S-2 fractions in both fish and chicken MYHC. The reactivity of fish skeletal myosin to chicken skeletal myosin monoclonal antibodies, and the localization of epitopes on the same myosin fraction, might be indicative of an evolutionary relationship between these two species: chicken and Tilapia nilotica.

The feasibility of employing monoclonal antibodies to detect important domains for functional and/or structural properties, has been showed by Wick et al. (1996), who found some evidence about the participation of the NA4 and 5C3 antibodies on the solubility of myosin in vitro at low ionic strength. Although EB165 and AB8 antibodies did not affect the solubility properties, or the morphology of myosin aggregates formed in low salt conditions it was suggested that domains responsible for these functions may be located outside the LMM domain. However preliminary studies could not to associate such antibodies with frozen storage stability of fish myosin obtained from Tilapia nilotica (data not shown).

Although abundant information exists concerning the different functional properties among fish species, the cause of such differences remains unclear. Because myosin is considered the principal protein responsible of the functional properties from different vertebrate muscles, it is possible that the use of monoclonal antibodies can be employed to correlate functionality and myosin structure from different species. A great advantage for food scientists is the existence of isolated monoclonal antibodies that are being employed for evolutionary relationship studies, and accordingly with this results they could be employed to determine the existence of structure-functionality relationship for fish myosins.

REFERENCES


