

High Performance Liquid Chromatography in the Analysis and Separation of Contractile Proteins

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Abstract

High performance liquid chromatography (HPLC) utilizing a support of small rigid particles of uniform size has been shown to provide a rapid method for separation of many types of molecules of biological interest. Myosin, the major component of the thick filaments of muscles, is a large asymmetric molecule containing two heavy chains molecular weight about 200000) and two each of two different classes of light chains (molecular weight range 16000-27000). Since myosin light and heavy chains are currently used as a useful markers in studying both diseased and normal muscle tissues, it is interesting to apply HPLC to the study of these proteins. Using a 330-Å-pore C-18 column we were able to separate each other myosin light chains. Furthermore, the light chains were digested with *S. aureus* V8 protease and the fragments obtained were separated on a 100-Å-pore C-18 column. By this approach we were able to demonstrate differences in the peptide maps of light chains characterized either by high degree of homology or by the same apparent molecular weight and apparent isoelectric point. The use of the HPLC chromatographic column filled with hydroxylapatite made possible the purification of the whole myosin molecule (molecular weight about 500000) in non denaturing conditions from relatively complex mixtures.

Key words: HPLC, hydroxylapatite, myosin, peptide mapping.

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The primary structure of proteins has long been thought to narrate the story of biological function. The first successful attempt at the elucidation of the amino-acid sequence of a biologically active protein was made by Sanger and co-workers in 1953 in their classical work on the insulin molecule. Since then many new techniques have facilitated the process of sequence determination.

A chronic problem faced by protein chemists has been the isolation of peptides resulting from enzymatic and/or chemical cleavage of larger molecules. This necessary step in sequence determination mandates the development of separation techniques which are sufficient to obtain peptides from small quantities of proteins. High performance liquid chromatography (HPLC) utilizing a support medium of small rigid particles of uniform size has been shown to provide a rapid method for separation of many types of small organic molecules [1]. HPLC has the potential to become an excellent alternative to conventional techniques of peptide fractionation.

Myosin, the major component of the thick filament of muscle, is a large asymmetric molecule containing two heavy chains (HC), molecular weight about 200000, and

two each of two different classes of light chains (LC), molecular weight range 16000-27000. Each heavy chain contains a long, fibrous "tail" region and a globular "head" which is associated with one type each of the two classes of light chains. Different muscles contain different myosin isoforms. These isoforms are currently used as markers of the transition occurring during embryonic, neonatal and adult stage of development and of the heterogeneity and plasticity of the muscle fibers and motor unit in adult animals.

In view of the emerging value of HPLC in the analysis and separation of peptides and proteins we have applied this technique to the study of vertebrate myosin.

Separation of Myosin Light Chain Mixtures

It is well known that the largest molecular weight of the peptides which can be analyzed by conventional (100-Å-pore diameter) reversed phase columns is about 2.000. Therefore myosin light chains cannot be separated by these columns. However it was recently demonstrated that pore diameter influences the resolution of reversed-phase columns [5,6]. Through the use of sup-

ports of 300 and 500 Å pore diameter it was shown that the resolution of high molecular weight collagens could be enhanced over that obtainable with a support of 100 Å pore diameter [5]. Using a macroporous support (300 Å) we were able to separate homologous myosin light chains characterized by a very similar and relatively high molecular weight by a acetonitrile gradient (see Fig. 1). Although the hydrophobicity of peptides increases generally with increasing molecular weight [8], the elution order of proteins does not correlate with molecular weight. In fact, we obtained the following order of elution for myosin light chains: LC1S (27000); LC3F (16000); LC1F (25000); LC2F (18000) and LC2S (20000). This is not surprising, as the dominant factor controlling biopolymer retention by hydrophobic interaction is the hydrophobic contact area between the solute and the packing. The distribution of hydrophobic residues in space and the number of residues that might interact with a surface are controlled not only by the primary but also by the secondary, tertiary and quaternary structure of the polymer. Because of these factors it is impossible to predict the order of elution of proteins from reversed-phase column (see ref. 7 for a review).

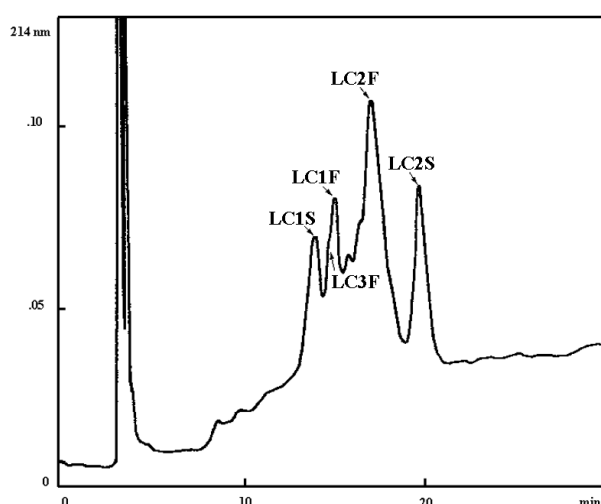


Figure 1. Reversed-phase chromatography of myosin light chains from mixed diaphragm rat muscle. Note that the myosin of diaphragm muscle is characterized by five light chains: three of the fast type (LCF) and two of the slow type (LCS). About 15 µg of light chains were loaded on a 330-Å-pore diameter C-18 column (Bakerbond TM). Solvents: (A) 40% acetonitrile in water containing 0.2% of trifluoroacetic acid (TFA); (B) 60% acetonitrile in water containing 0.2% TFA. Following sample application the proteins were eluted at 1.0 ml/min with a linear gradient from 0 to 20% of solvent B over 5 min and from 20 to 100% solvent B over 25 min. The eluate was monitored at 214 nm.

Fractionation of Peptides Mixtures: Peptide Mapping of Myosin Light Chains

To test the analytical power of reversed-phase HPLC in peptide mapping of myosin light chains, we have fragmented with the *S.aureus* V8 protease two very homologous light chains, i.e. LC1F and LC3F from rabbit myosin. The digests were analyzed by reversed-phase chromatography on 100-Å-pore C-18 column (Fig. 2). Sequences studies [3] have shown that the extent of

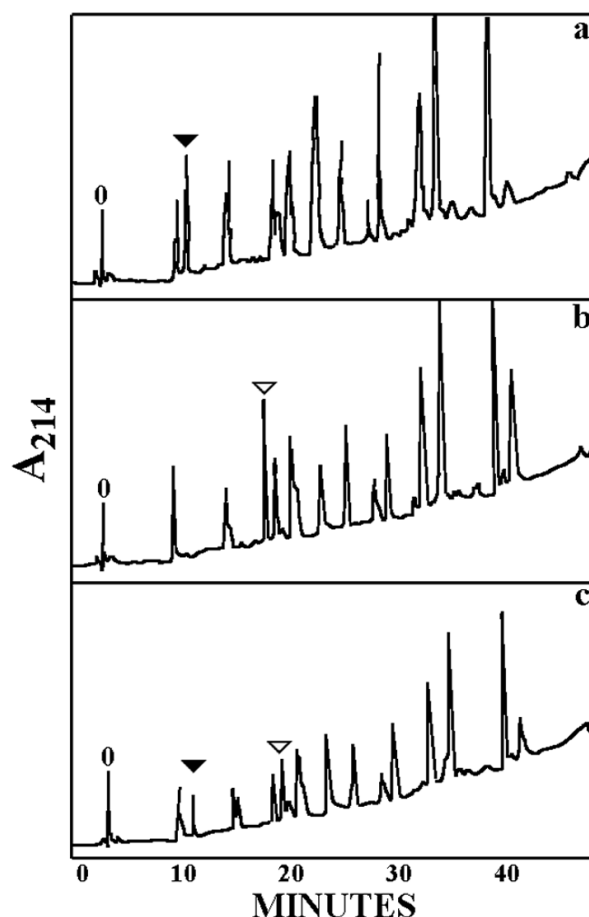


Figure 2. Liquid chromatographic maps of rabbit myosin light chains. Myosin light chains were incubated in 50 mM sodium phosphate buffer (pH 7.8) with *S. aureus* V8 protease (1:500, mol/mol) for 16 h. A 50 min linear gradient from 0 to 50% of acetonitrile in 20 mM potassium phosphate buffer (pH 5.9) was begun immediately upon injection of the digest. A 30 µg amount of protein was injected, except in (c) where a mixture of 10 µg of LC1F and 10 µg of LC3F was injected. The flow-rate was 1.0 ml/min. A µBonbapack C-18 column (Waters) was used. The peak marked 0 is present in all maps and is due to the digestion buffer. Peptides indicated by filled triangles are unique to the LC1F map and that indicated by open triangle is unique to the LC3F map. Key: (a) LC1F; (b) LC3F; (c) LC1F + LC3F.

homology is very great between this two light chains, the main distinguishing feature of LC1F being an additional 41 residues of LC3F containing five aminoacid replacements when compared with the corresponding sequence of LC1F. Other than these differences, the aminoacid sequences appear to be identical. At first sight it appears that the maps of LC1F and LC3F are very similar. However, a more detailed inspection of the two profiles revealed some distinct differences. In fact, at least one peak for each LC1F and LC3F in their maps is unique. To be sure that these peaks are really different in the two maps we have performed the co-analysis of the two digests (Fig. 2c). It is evident that the peak marked with a solid triangle is distinctive for the LC3F map, whereas that marked with an open triangle is distinctive for the LC3F map.

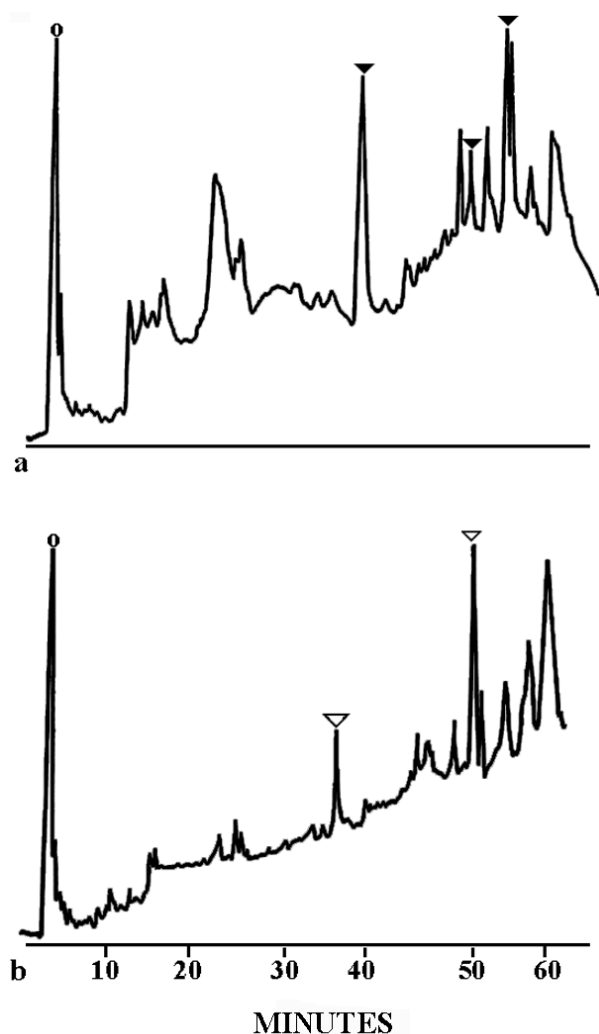


Figure 3. Liquid chromatographic maps of rat myosin light chains. The conditions of peptides generation and of peptide separation are the same as in Fig.2. Peptides indicated by filled triangles are unique to the LC1F map and those indicated by open triangles are unique to the LC3F map. Key: (a) LC1F; (b) LC3F.

The sequences of rat LC1F and LC3F are not yet available, even though they are supposed to be as homologous as in the rabbit. The hypothesis seems corrected as the peptide mapping performed with HPLC reveals many similarities between LC1F and LC3F from rat myosin (Fig. 3).

The digestion patterns of myosin light chains are highly reproducible under fixed experimental conditions such as amount of protease, temperature, digestion time and digestion buffer. Peptide mapping with HPLC was also used for demonstrating structural differences in myosin light chains characterized by the same apparent molecular weight and apparent isoelectric point [2].

Purification of Myosin Molecule

Certain proteins and peptides from complex sources, such as membranes and viruses, seemed to possess a great affinity for the support materials, frequently binding irreversibly. Since all the supports used were silica or glass based material it could be reasoned that the peptides were either adhering to free silanol groups or strong hydrophobic interactions were occurring between the packing and protein. Unfortunately this is also the case of myosin molecule. However, recently a new type of column packed with hydroxylapatite has been introduced. Hydroxylapatite offers unique selectivities and can be very valuable for separating proteins which are not well resolved by other methods. Protein-hydroxylapatite interactions are a function of the net charge on the protein, whether acidic or basic [4]. We

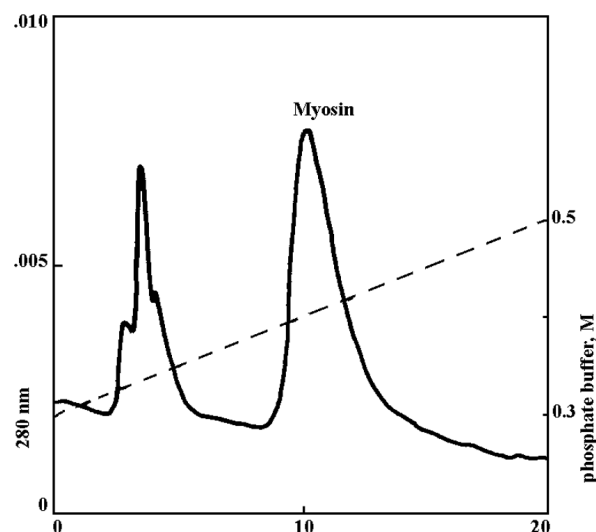


Figure 4. myosin purification with hydroxylapatite column (HPHT, Bio Rad). About 100 μ g of crude myosin was loaded in the column. Solvents: A 0.3 M sodium phosphate buffer (pH 7.0); B 0.5 M sodium phosphate buffer (pH 7.0). The proteins were eluted at 1.0 ml/min with a linear gradient from 0 to 100% B over 20 min. The eluate was monitored at 280 nm.

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have used this column to purify the whole myosin molecule (i.e. heavy chains + light chains) from a complex mixture of a proteins in non-denaturing conditions (Fig.4). Such a column can be utilized successfully for purify myosin from those biological tissues in which classical methods appear unsuitable to obtain a degree of purity sufficient for enzymatic and structural studies.

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