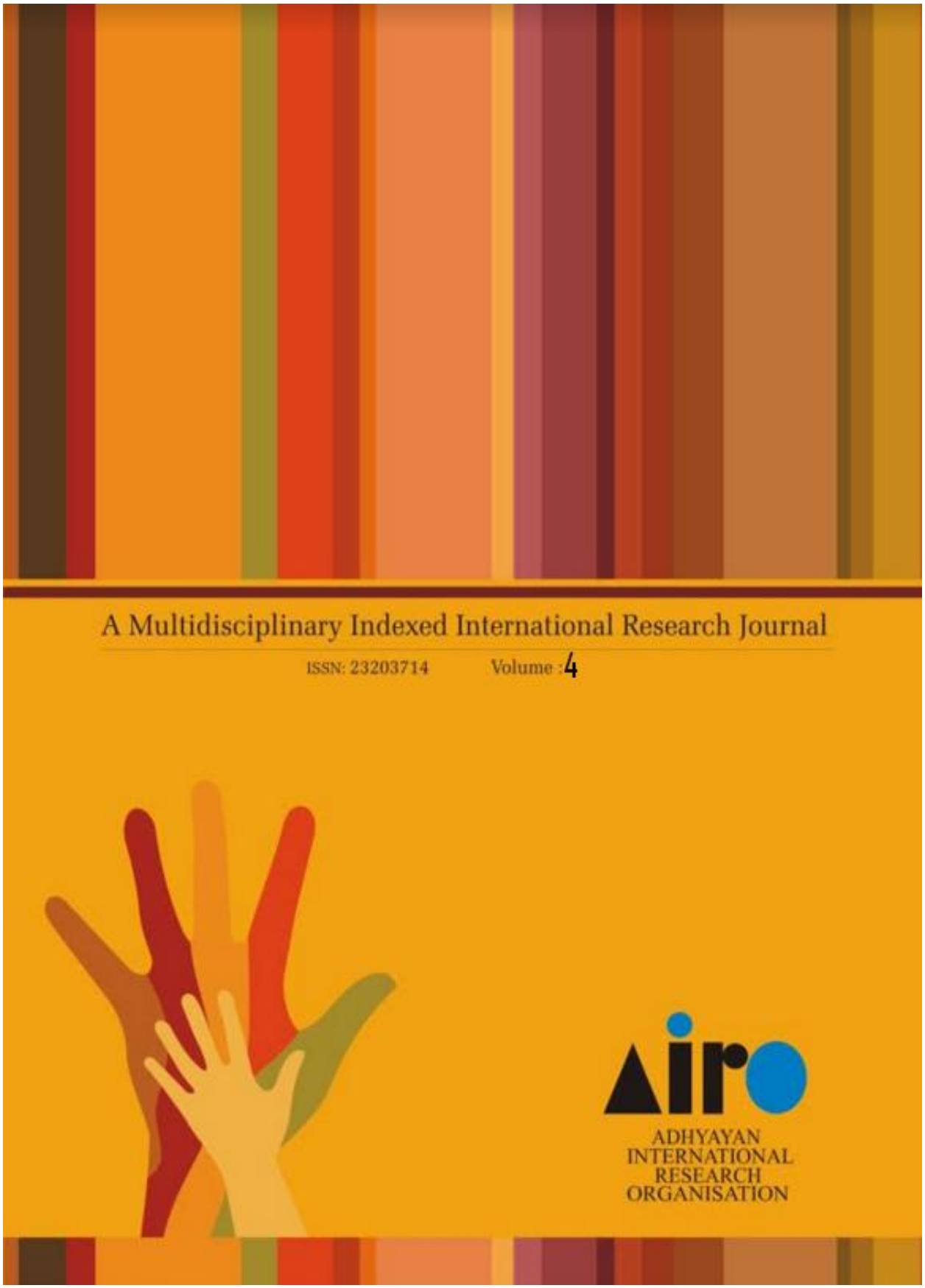
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An Overview of the Analytical Methods of High Pressure Liquid Chromatography

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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 type 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.

Introduction

The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically micro liters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are a function of specific physical interactions with the adsorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the

mobile phase. The time at which a specific analytic elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is an identifying characteristic of a given analytic.

Many different types of columns are available, filled with adsorbents varying in particle size, and in the nature of their surface ("surface chemistry"). The use of smaller particle size packing materials requires the use of higher operational pressure ("backpressure") and typically improves chromatographic resolution (*i.e.* the degree of separation between consecutive analytes emerging from the column). Sorbent particles may be hydrophobic or polar in nature.

Common mobile phases used include any miscible combination of water with various organic solvents (the most common are acetonitrile and methanol). Some HPLC techniques use water-free mobile phases (see Normal-phase chromatography below). The aqueous component of the mobile phase may contain acids (such as formic, phosphoric or trifluoroacetic acid or salts to assist in the separation of the sample components. The composition of the mobile phase may be kept constant ("isocratic elution mode") or varied ("gradient elution mode") during the chromatographic analysis. Isocratic elution is typically effective in the separation of sample components that are very different in their affinity for the stationary phase. In gradient elution the composition of the mobile phase is varied typically from low to high eluting strength. The eluting strength of the mobile phase is reflected by analyte retention times with high eluting strength producing fast elution (=short retention times). A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile (in water or aqueous buffer) and progress linearly to 95% acetonitrile over 5–25 minutes. Periods of constant mobile phase composition may be part of any gradient profile. For example, the mobile phase composition may be kept constant at 5% acetonitrile for 1–3 min, followed by a linear change up to 95% acetonitrile.

A rotary fraction collector collecting HPLC output. The system is being used to isolate a fraction containing Complex I from *E. coli* plasma membranes. About 50 litres of bacteria were needed to isolate this amount.

The chosen composition of the mobile phase (also called eluent) depends on the intensity of interactions between various sample components ("analytes") and stationary

phase (*e.g.* hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases analytes partition between the two during the separation process taking place in the column. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/acetonitrile gradient, more hydrophobic components will elute (come off the column) late, once the mobile phase gets more concentrated in acetonitrile (*i.e.* in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depends on the nature of the column and sample components. Often a series of trial runs is performed with the sample in order to find the HPLC method which gives adequate separation.

Prolactin is a hormone produced by the anterior pituitary. There are multiple circulating forms: monomeric prolactin (molecular weight _23 kDa), the predominant form in healthy people; big prolactin (_60 kDa); and big-big or macroprolactin (_150 kDa), a complex of monomeric prolactin and an antiprolactin autoantibody, usually IgG.1,2 Macroprolactin accounts for a small but variable percentage of circulating prolactin and also reacts variably with different assays, thus contributing to the large variation in the prolactin reference intervals of immunoassay platforms.1 Macroprolactin is thought not to be bioavailable and does not cause clinical effects or symptoms.3 Macroprolactin concentration in the serum may be elevated (causing macroprolactinaemia) due to the reduced clearance of the prolactin–IgG complex, rather than increased prolactin production. 4 Macroprolactinaemia is estimated to account for up to 26% of all cases of elevated total prolactin.1 However, the incidence of macroprolactinaemia may depend on how it is defined and the prolactin assay used.3 Gel filtration chromatography (GFC) is regarded as the reference method for separation and quantification of the different prolactin forms.3 However, this method is complex, time-consuming and does not lend itself to routine application.1 Polyethylene glycol (PEG) precipitation is the most widely used method to screen for macroprolactin in routine practice. However, there are issues with the use of PEG, including positive interference with some immunoassay platforms leading to recoveries in excess of 100%.3 We developed a size exclusion high pressure liquid chromatography (HPLC)

method to rapidly separate the different prolactin forms. 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 aminoacid 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 facilitate 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 uniforms 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 is forms. These is forms 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.

History and development

Prior to HPLC scientists used standard liquid chromatographic techniques. Liquid chromatogra-

chromatographic systems were largely inefficient due to the flow rate of solvents dependent on gravity. Separations took many hours, and sometimes days to complete. Gas chromatography (GC) at the time was more powerful than (LC), however, it was believed that gas phase separation and analysis of very polar high molecular weight biopolymers was impossible. GC was ineffective for many biochemists because of the thermal

instability of the solutes. As a result, alternative methods were hypothesized which would soon result in the development of HPLC.

Following on the seminal work of Martin and Synge in 1941, it was predicted by Cal Giddings, Josef Huber, and others in the 1960s that LC could be operated in the high-efficiency mode by reducing the packing-particle diameter substantially below the typical LC (and GC) level of 150 μm and using pressure to increase the mobile phase velocity. These predictions underwent extensive experimentation and refinement throughout the 60s into the 70s. Early developmental research began to improve LC particles, and the invention of Zipax, a superficially porous particle, was promising for HPLC technology.

The 1970s brought about many developments in hardware and instrumentation. Researchers began using pumps and injectors to make a rudimentary design of an HPLC system. Gas amplifier pumps were ideal because they operated at constant presser and did not require leak free seals or check valves for steady flow and good quantitation. Hardware milestones were made at Dupont IPD (Industrial Polymers Division) such as a low-dwell-volume gradient device being utilized as well as replacing the septum injector with a loop injection valve.

While instrumentational developments were important, the history of HPLC is primarily about the history and evolution of particle After the introduction of porous layer particles, there has been a steady trend to reduced particle size to improve efficiency. However, by decreasing particle size, new problems arose. The practical disadvantages stem from the excessive pressure drop needed to force mobile fluid through the column and the difficulty of preparing a uniform packing of extremely fine materials. Every time particle size is reduced significantly, another round of instrument development usually must occur to handle the pressure

Analytical Methods of High Pressure Liquid Chromatography

A reservoir holds the solvent [called the mobile phase, because it moves]. A high-pressure pump [solvent delivery system or solvent manager] is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute. An injector [sample manager or autosampler] is able to introduce [inject] the sample into the continuously flowing mobile phase stream

that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. A detector is needed to *see* the separated compound bands as they elute from the HPLC column [most compounds have no color, so we cannot see them with our eyes]. The mobile phase exits the detector and can be sent to waste, or collected, as desired. When the mobile phase contains a separated compound band, HPLC provides the ability to collect this fraction of the eluate containing that purified compound for further study. This is called preparative chromatography [discussed in the section on HPLC Scale].

Note that high-pressure tubing and fittings are used to interconnect the pump, injector, column, and detector components to form the conduit for the mobile phase, sample, and separated compound bands.

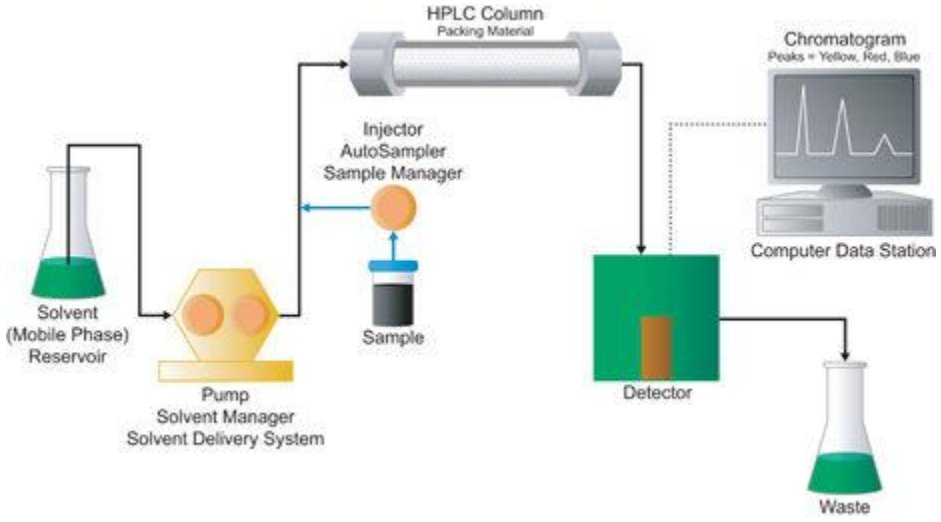

Figure : High-Performance Liquid Chromatography [HPLC] System

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 supports 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).

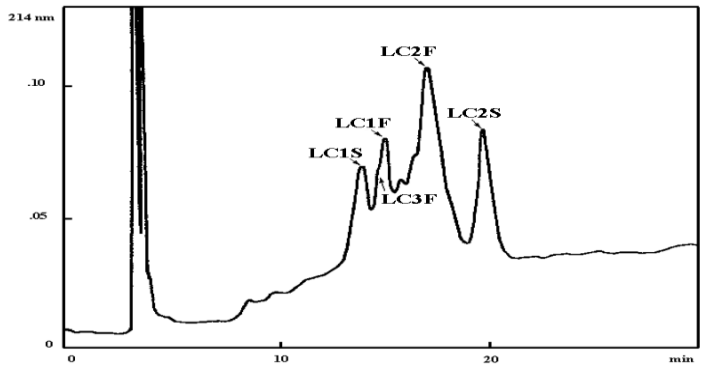

Figure 1.

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

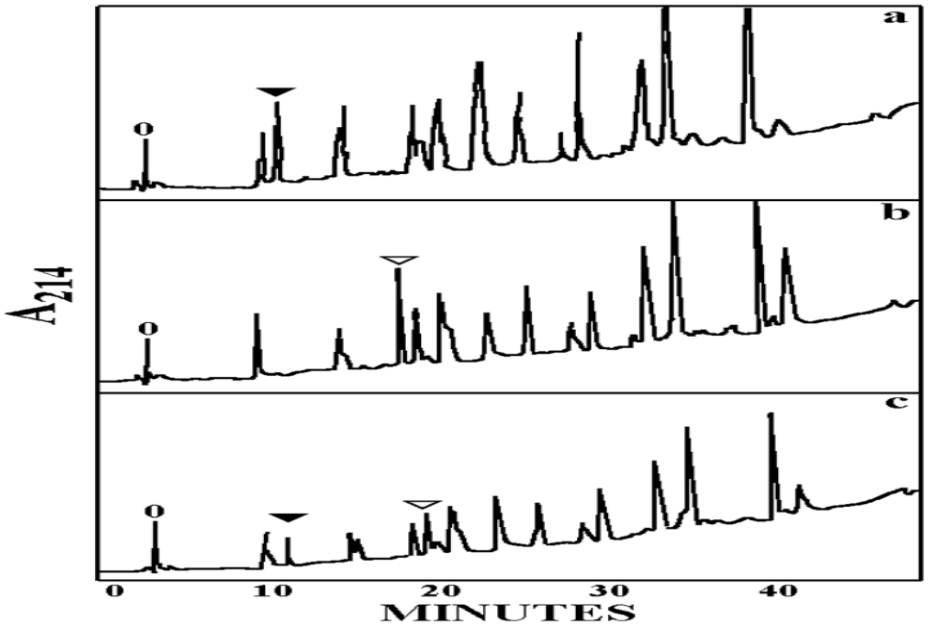

Figure 2.

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) were 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.

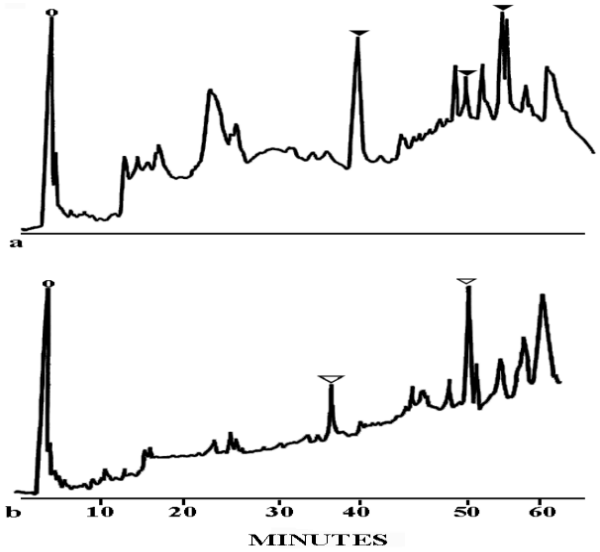

Figure 3.

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 ss 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

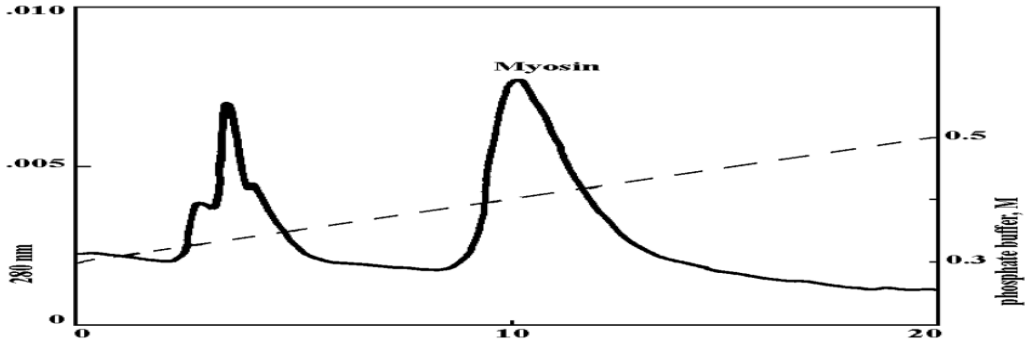

Figure 4.

Myosin purification with hydroxylapatite column (HPHT, Bio Rad). About 100 μg 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.

have used this colum 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.

ANALYSIS

A simple way to understand how we achieve the separation of the compounds contained in a sample is to view the diagram in Figure.Mobile phase enters the column from the left, passes through the particle bed, and exits at the right. Flow direction is represented by green arrows. First, consider the top image; it represents the column at time zero [the moment of injection], when the sample enters the column and begins to form a band. The sample shown here, a mixture of yellow, red, and blue dyes, appears at the inlet of the column as a single black band. [In reality, this sample could be anything that can be dissolved in a solvent; typically the compounds would be colorless and the column wallopaque, so we would need a detector to see the separated compounds as they elute.After a few minutes [lower image], during which mobile phase flows continuously and steadily past

the packing material particles, we can see that the individual dyes have moved in separate bands at different speeds. This is because there is a competition between the mobile phase and the stationary phase for attracting each of the dyes or analytes. Notice that the yellow dye band moves the fastest and is about to exit the column. The yellow dye likes [is attracted to] the mobile phase more than the other dyes. Therefore, it moves at a *faster* speed, closer to that of the mobile phase. The blue dye band likes the packing material more than the mobile phase. Its stronger attraction to the particles causes it to move significantly *slower*. In other words, it is the most retained compound in this sample mixture. The red dye band has an intermediate attraction for the mobile phase and therefore moves at an *intermediate* speed through the column. Since each dye band moves at different speed, we are able to separate it chromatographically.

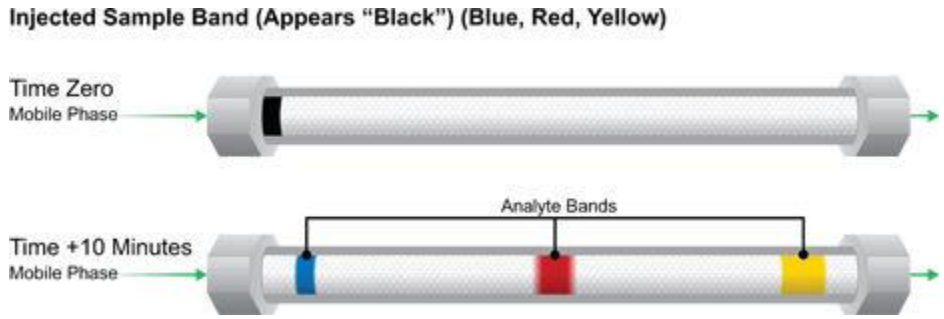

Figure : Understanding How a Chromatographic Column Works – Bands

Applications of HPLC

HPLCs can be used in the following applications:

- Water purification
- Preconcentration of trace components
- Ligand-exchange chromatography
- Ion-exchange chromatography of proteins
- High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

Benefits of HPLC

The key benefits of HPLC systems are as follows:

- Controls and automates chromatography instrumentation
- Provides data management, security features, and reporting and instrument validation.
- Powerful and adaptable
- Increases productivity by managing all the areas of analysis - from sample to instrument, and from separation to reporting results.
- Affordable

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