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Note

Separation of myosin light chains by reversed-phase high-performance liquid chromatography on wide pore supports

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The application of reversed-phase chromatography to peptide separations has revolutionized peptide chemistry. Single amino acid substitution is usually detectable in peptides containing less than 20 amino acids. However, it is worth noting that there may be broad differences in the way various reversed-phase packings separate polypeptides. These differences become particularly prominent in the higher molecular weight hydrophobic peptides.

Recently, it was demonstrated that pore diameter influences the resolution of reversed-phase columns[1,2]. 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[1]. In view of the increasing value of wide-pore supports in reversed-phase liquid chromatography of biopolymers[3] and of our interest in the characterization of the light chains associated with myosin from various types of tissues, we decided to adapt this method to the separation of myosin light chains from fast and slow muscles of the rat. These proteins are characterized by a molecular weight ranging from 27,000 to 16,000 daltons. In particular, light chains (LC) of fast (F) muscles are LC1F, LC2F and LC3F (25,000; 18,000 and 16,000 daltons, respectively), while the light chains of slow (S) muscles are LC1S and LC2S (27,000 and 25,000 daltons, respectively)[4,5].

In this paper it will be shown that these proteins are separated in less than 30 min using a wide-pore (330 Å) reversed-phase column. The results also show that reversed-phase liquid chromatography makes possible the resolution of homologous polypeptides characterized by a similar molecular weight in a submilligram sample and greatly facilitates the analysis of biopolymers available in only small amounts.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC grade) and trifluoroacetic acid (TFA) were obtained from either BDH Chemicals (Poole, U.K.) or Baker (Deventer, The Netherlands). Water was glass-distilled and filtered through a 0.45-μm Millipore filter. Mobile phase solutions were degassed by sonication before use.

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Purification of myosin light chains

Myosin was purified from rat muscles according to Dalla Libera *et al.*[6]. We found it important to include protease inhibitors, pepstatin and phenylmethane sulphonylfluoride, in order to avoid degradation of proteins. The proteolysis was further reduced when myosin was extracted in the presence of 5 m*M* ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. This is probably due to the inhibition of some Ca-dependent protease present in muscles[7]. The muscles used in this study were the fast-contracting Extensor Digitorum Longus (EDL), the slow-contracting soleus and the diaphragm with intermediate properties. In early experiments myosin light chains were isolated from rat muscles as reported earlier[8]. Subsequently, however, we developed a faster electrophoretic technique that allows a good recovery of proteins from gels. Briefly, about 10 mg of myosin were subjected to electrophoresis on a 3-mm thick slab gel consisting of 7.5% acrylamide according to Laemmli[9]. When the dye front reached the bottom of the slab the electrophoresis was stopped. The strip of the slab corresponding to the dye front, containing all the light chains, was cut and equilibrated for 1 h in a large volume of 20 m*M* Tris–acetate (pH 8.1). The strip was then cut into small pieces, which were placed in an apparatus for electrophoretic elution of proteins from gels (ISCO Model 1750)[10]. The elution was carried out overnight in 20 m*M* Tris–acetate (pH 8.1) at a power of 1 W. The eluted light chains were collected and stored in small test-tubes at −20°C. The purity of each preparation was tested by sodium dodecyl sulphate (SDS) gel electrophoresis and by staining with the silver technique described by Merrill *et al.*[11].

Apparatus

A Perkin-Elmer high-performance liquid chromatographic system was used as described previously[12]. A Bakerbond TM C-18 wide-pore (330 Å) column (5 μm; 25 cm × 4.6 mm I.D.) was obtained from Baker (Phillipsburg, NJ, U.S.A.). The drift of the baseline due to the acetonitrile gradient used for elution was corrected by a Perkin-Elmer LC Autocontrol apparatus.

HPLC method

All chromatographic runs were carried out at room temperature at a flow-rate of 1.0 ml/min. The column was equilibrated with solvent A, consisting of acetonitrile–water (40:60) to which was added 0.2% (w/v) of TFA. Light chains were eluted with linear gradient segments of solvent B, consisting of acetonitrile–water (60:40) to which was added 0.2% (w/v) of TFA as follows: 0 to 20% B in 5 min, then to 100% B in 25 min. The effluent was monitored at 214 nm. A 15-min washing step with concentrated acetonitrile, a 5-min reverse linear gradient and a short re-equilibration period were necessary for satisfactory reproducibility.

Electrophoretic analysis of the chromatographed peptides was carried out according to Laemmli[9] using a 15% acrylamide concentration. The gels were stained with the silver technique[11].

RESULTS

Fig. 1b shows the electrophoretic pattern of the myosin light chains from rat soleus muscle, prepared following the protocol described by Biral *et al.*[8], stained with

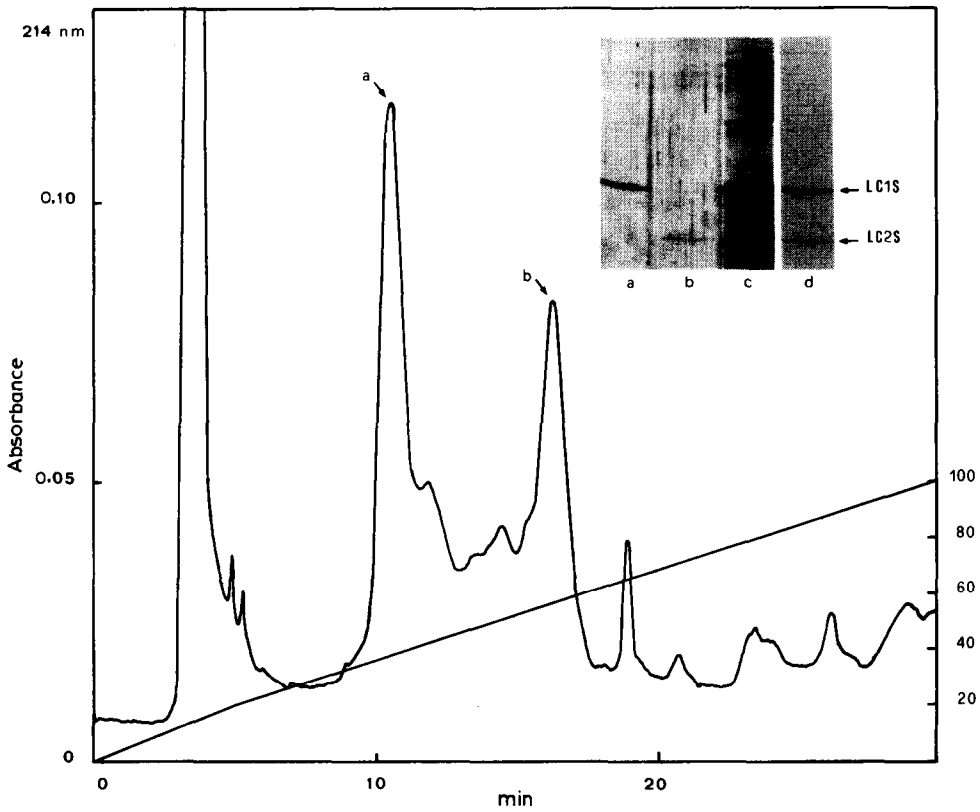

Fig. 1. Reversed-phase chromatography of myosin light chains from rat soleus slow muscle. About 15 μg of light chains were loaded on a 330 Å pore diameter C_{18} column. Solvents: (A) 40% acetonitrile in water containing 0.2% (w/v) of TFA; (B) 60% acetonitrile in water containing 0.2% (w/v) of TFA. Following 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. Inset: SDS gel electrophoresis on 15% acrylamide of starting material (SM) and selected fractions of the chromatography in Fig. 1. (a) First eluted peak; (b) second eluted peak; (c) SM (5 μg) stained with silver; (d) SM (5 μg) stained with Coomassie blue.

Coomassie blue. Two main bands are present, corresponding to LC1S (27,000 daltons) and LC2S (20,000 daltons). However, by staining the slab with silver[11] several additional bands are revealed (see inset in Fig. 1, lane c). When this sample is analysed by HPLC the chromatogram shown in Fig. 1 is obtained. Two well resolved peaks and other minor components are present. Taking into account the lack of complete purity of the light chain preparation, the minor peaks are probably due to impurities. The effluents corresponding to the two main peaks were collected and subjected to gel electrophoresis. Each fraction corresponds to a myosin light chain, and in particular the first eluted peak represents LC1S while the second eluted peak represents LC2S (Fig. 1, peaks a and b, respectively). Thus, with decreasing molecular weight proteins are relatively more retarted.

Fig. 2 shows a typical preparation of EDL myosin and of its three light chains

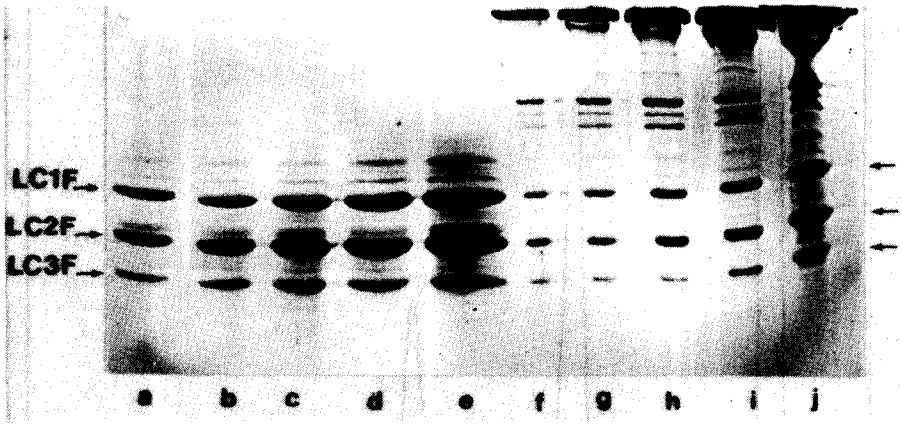

Fig. 2. SDS gel electrophoresis of myosin and its light chains from rat EDL fast muscle. (a)–(e) Increasing amounts of myosin light chain sample prepared by the electrophoretic method described under Experimental; (f)–(j) increasing amounts of original myosin sample. The gel was stained with Coomassie blue.

isolated by the electrophoretic method described here. It is evident that the purity of myosin light chains is comparable to that obtained with the more laborious method involving the dissociation of myosin subunits with urea (see Fig. 1). By this method it is possible to concentrate in the electrophoretic front all the light chain material in few hours, thus reducing proteolytic events. We think that this electrophoretic method could be used for the purification of other proteins, choosing appropriate acrylamide concentrations. When EDL light chains are analysed by HPLC the profile shown in Fig. 3 is obtained. Two peaks and a shoulder are evident as the major components of the chromatogram. The electrophoretic pattern of the collected fractions is shown in the inset in Fig. 3. The first eluted peak represents LC1F (25,000 daltons), while the second peak represents LC2F (18,000 daltons). The shoulder eluting just before the first peak is LC3F (16,000 daltons) (see insert in Fig. 3, lanes b, c and a, respectively). The fact that LC3F elutes near LC1F is not surprising, as these two proteins are so homologous that only a few peptides are unique in their proteolytic maps when the digests are analysed by the reversed-phase method on the C_{18} column[12].

When a mixture of light chains from EDL and soleus is analysed the profile shown in Fig. 4 is obtained. It is evident that the light chains from fast myosin are resolved from those of slow myosin. To substantiate this result we chromatographed the light chains obtained from the myosin of a naturally mixed muscle, *viz.* rat diaphragm[13]. This myosin is characterized by five light chains: three fast and two slow components[14]. The result is shown in Fig. 5. With the only exception that LC3F is poorly resolved from LC1F, we were able to reproduce the pattern presented in Fig. 4. In fact, the slow light chains are again well resolved from the fast light chains (see Fig. 5).

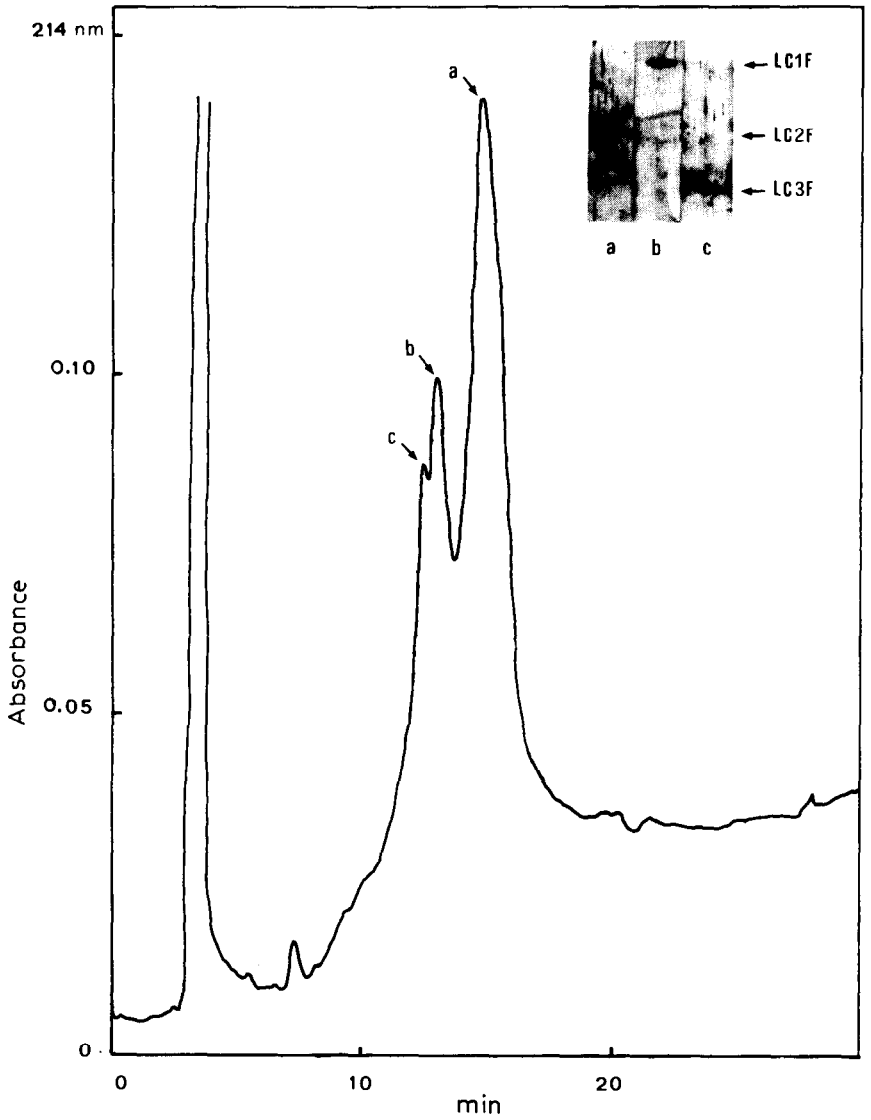

Fig. 3. Reversed-phase chromatography of myosin light chains from EDL rat fast muscle. Conditions of chromatography as in Fig. 1. About 20 μg of light chains were analysed. Inset: SDS gel electrophoresis on 15% acrylamide of selected fractions of the chromatography in Fig. 3. (a) Third eluted peak; (b) second eluted peak; (c) first eluted peak. The slab was stained with silver.

DISCUSSION

Reversed-phase high-performance liquid chromatography is a powerful tool for the separation of proteins and peptides[15]. Because protein separation is based on differences in hydrophobicity, this technique was suggested as a means of detecting neutral amino acid substitution in proteins. In this study we have shown that, using

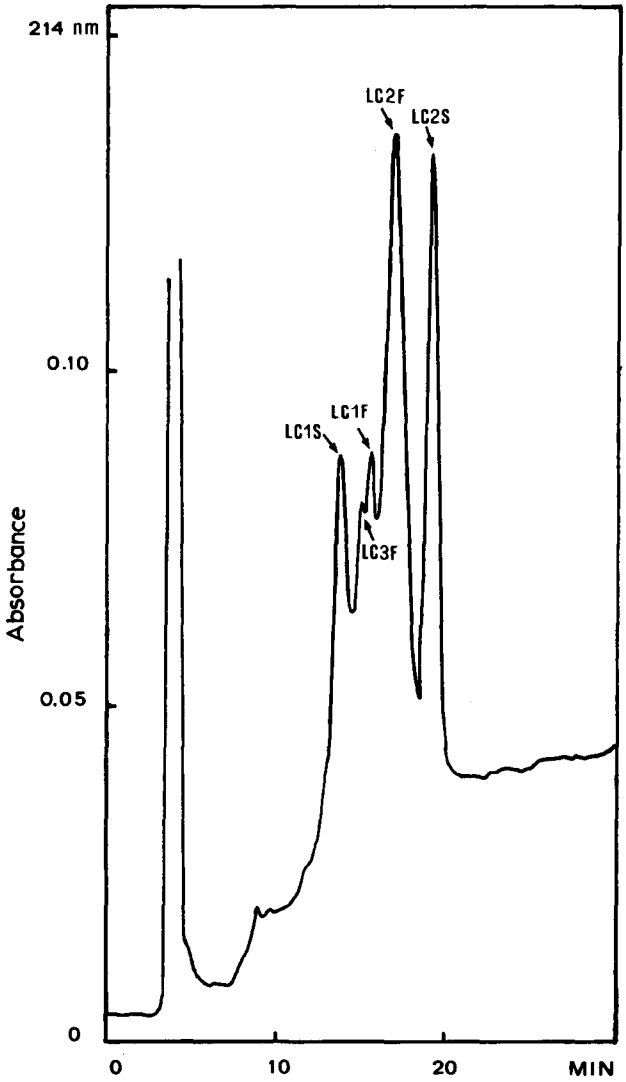

Fig. 4. Reversed-phase chromatography of myosin light chains. A mixture containing about the same amount (20 μg each) of myosin light chains from both fast EDL and slow soleus rat muscles was analysed as in Fig. 1. Proteins were prepared by the electrophoretic method described under Experimental.

a macroporous support (330 Å), it is possible to separate homologous proteins characterized by a very similar and relatively high molecular weight. Although the hydrophobicity of peptides increases generally with increasing molecular weight[16], 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 (27,000), LC3F (16,000), LC1F (25,000), LC2F (18,000) and LC2S (20,000 daltons). 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

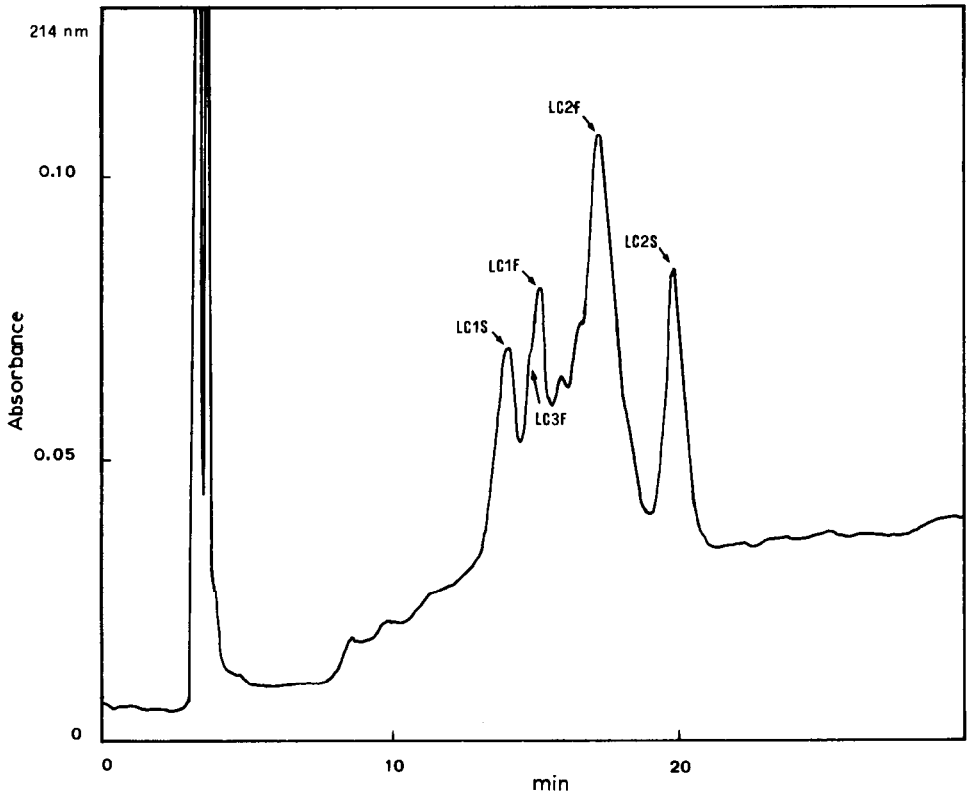

Fig. 5. Reversed-phase chromatography of myosin light chains from mixed diaphragm rat muscle. About 20 μg of myosin light chains were analysed as in Fig. 1. The proteins were prepared by the electrophoretic method described under Experimental.

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 not possible to predict the order of elution of proteins from reversed-phase columns (see ref. 3 for a review). The use of acids in the mobile phase has been found to increase both the recovery and the resolution of many proteins. TFA is the most popular because it is an excellent solubilizing agent and allows the detection of peptide bonds below 230 nm[17]. However, the use of such an aggressive mobile phase generally disrupts the three-dimensional structure of most biopolymers, with the risk of cleavage of some covalent bonds. This is not the case with myosin light chains. When we subjected the chromatographic fractions to electrophoresis we found only undegraded light chain material. Therefore, the method described here is suitable for separating rapidly myosin light chains from different muscles, discriminating small differences in amino acid composition. This approach may be particularly useful when the proteins are available in such a reduced amount that peptide mapping and analysis of derived fragments by high-performance liquid chromatography are not possible.

We are now attempting to extend the applicability of this method to the study of those myosin light chains which are characterized by similar molecular weights and isoelectric points, in order to understand if they are very homologous but nevertheless products of different genes or if they are products of the same gene.

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