Note

Peptide mapping of myosin light chains by high-performance liquid chromatography

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(Received March 3rd, 1983)

In view of the emerging value of reversed-phase liquid chromatography in peptide mapping analysis[1–3] and of our interest in structural relationships between types of light chains associated with skeletal muscle myosin[4–7], we decided to adapt this method to the analysis of light chains of rabbit fast-muscle myosin. The light chains associated with skeletal muscle myosin of vertebrate fast muscles are well characterized chemically, immunologically and functionally. They are classified into two main classes[8]: alkali light chains (A1 or LC1F, molecular weight 25,000, and A2 or LC3F, molecular weight 16,000) and DTNB light chain (LC2F or P light chain, molecular weight 18,000). Sequence studies[9] have shown that the extent of homology is greatest between LC1F and LC3F, the main distinguishing feature of LC1F being an additional 41 residues at its N-terminal end (difference peptide) not present in LC3F. Further, the eight N-terminal residues of LC3F contain five amino acid replacements compared with the corresponding sequence of LC1F. Other than these differences, the amino acid sequences appear to be identical. Analysis of LC2F sequence has shown that it is related to the alkali light chains, displaying a similar three-dimensional structure. In spite of this similarity, an average of only one of four residues are identical in their respective primary structure[10].

In this paper it will be shown that, as expected from the known primary structures, large differences exist between the peptide maps of LC1F and LC3F and the map of LC2F, when the digests are analysed on a reversed-phase liquid chromatographic column. In contrast, only a few peptides distinguish the proteolytic map of LC1F and LC3F.

Finally, the results reported here show that reversed-phase liquid chromatography can be used successfully as a rapid and reliable procedure for revealing unambiguous differences even in the peptide maps of very homologous proteins such as LC1F and LC3F rabbit myosin light chains. This system makes possible the resolution of peptides in a submilligram sample and greatly facilitates the structural analysis of proteins available in only small amounts.

EXPERIMENTAL

Reagents

Acetonitrile (HPLC grade) was obtained from either Carlo Erba (Milan, Italy)

or E. Merck (Darmstadt, G.F.R.). Water was glass-distilled and filtered. *Staphylococcus aureus* V_8 protease was obtained from Miles Labs. (Slough, Great Britain). All other reagents were of analytical-reagent grade. The mobile phase solutions were passed through a 0.45-μm Millipore filter and degassed by sonication before use.

Purification of myosin light chains

Individual myosin light chains were isolated from rabbit adductor skeletal muscles as reported in the immunological study of Biral *et al.*[11]. The purity of each light chain preparation was tested by sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to Laemmli[12]. To confirm further the high purity of the preparations, gels were stained by the highly sensible silver staining method described by Merril *et al.*[13] (not shown).

Preparation of proteolytic peptides

Cleavage of individual myosin light chains with *S. aureus* V_8 protease was performed either in 20 mM Tris–acetate buffer (pH 8.1) or in 50 mM sodium phosphate buffer (pH 7.8) as indicated in the legends to the figures. The ratio of protease to light chain used was 1:500 (mol/mol) and digestion was allowed to proceed for 2–16 h at 25°C.

Apparatus

A Perkin-Elmer high-performance liquid chromatographic system was used. This consisted of a Series 3B liquid chromatograph equipped with a Model LC 75 variable-wavelength UV detector and a Rheodyne Model 7105 sample injector with a 175-μl sample loop. All chromatograms were recorded and analysed using a Perkin-Elmer Sigma 10 chromatography data station. A μBondapack C_{18} column (10 μm, 30 cm × 3.9 mm I.D.) was obtained from Waters Assoc. (Milford, MA, U.S.A.). A Whatman guard column packed with Co:Pell ODS (30–38 μm glass beads) was used for all analyses.

HPLC method

All chromatographic runs were carried out at room temperature at a flow-rate of 1.0 ml/min. A linear gradient from 0 to 50% of acetonitrile in 20 mM potassium phosphate (pH 5.9) in 50 min was begun immediately upon injection of the sample. Direct detection of peptide bonds was carried out at 214 or 215 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.

RESULTS

In order to determine the time required for complete digestion and for optimal generation of appropriate peptides, the progress of digestion of LC3F rabbit myosin light chain as a function of time was followed by liquid chromatography mapping. The results are shown in Fig. 1. After 2 h (Fig. 1a) a number of peptides become evident. Fig. 1 also shows the changes that occur with increasing digestion time. In fact, the profiles obtained after 4, 8 and 16 h of incubation are very different from the relatively simple one obtained after 2 h. Moreover, the maps resulting after 8 and 16 h
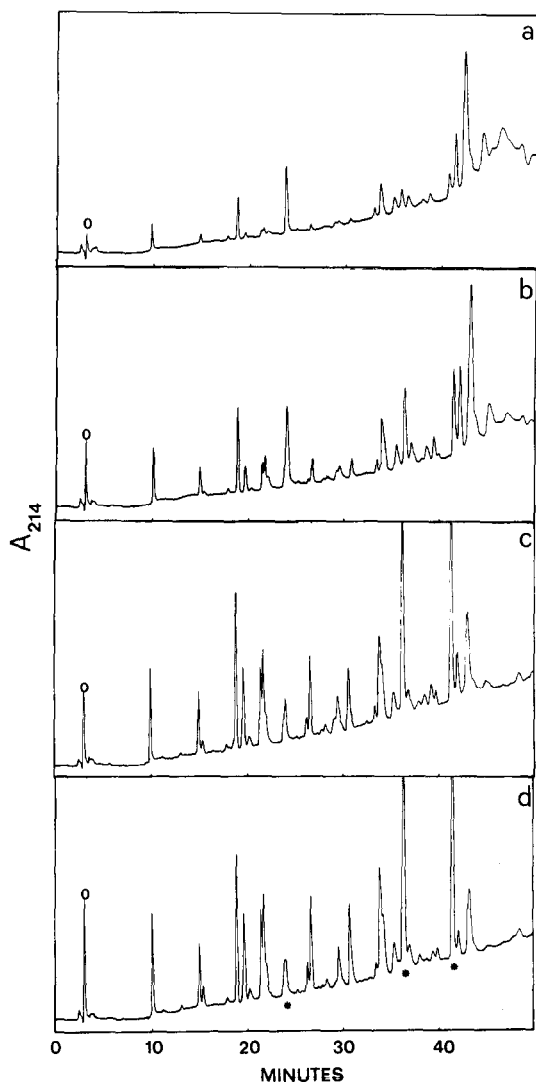
Fig. 1. Analytical liquid chromatographic maps showing the time course of digestion of LC3F rabbit myosin light chain with *S. aureus* V_8. The profiles were obtained after (a) 2 h, (b) 4 h, (c) 8 h and (d) 16 h of digestion. The protease to protein ratio was 1:500 (mol/mol); the digestion buffer was 50 mM sodium phosphate (pH 7.8). For each run 30 μg (about 1 nmol) of protein digest were injected and a linear gradient from 0 to 50% of acetonitrile in 20 mM potassium phosphate buffer (pH 5.9) over a period of 50 min was begun immediately upon injection of the digest. A μBondapak C_{18} column was used. The flow-rate was 1.0 ml/min. The peak marked 0 is present in all maps and is due to the digestion buffer. In (d) asterisks indicate the tyrosine-containing peptides identified by a parallel run monitored at 280 nm.

are very similar, suggesting that the proteolytic reaction has reached a steady-state equilibrium. The time course of LC1F and LC2F myosin light chains showed a very similar behaviour of proteolytic digestion (results not shown). In Fig. 1d the tyrosine-containing peptides, identified with a parallel run monitored at 280 nm, are indicated with asterisks.

The peak emerging at 3 min (peak 0) is due to the digestion buffer. The un-digested LC3F is not eluted under these conditions.

In Fig. 2a and b are shown, for comparison, the patterns obtained after 16 h of digestion of the very homologous rabbit myosin light chains LC1F and LC3F. At first sight it appears that the maps of LC1F and LC3F are very similar. However, a more
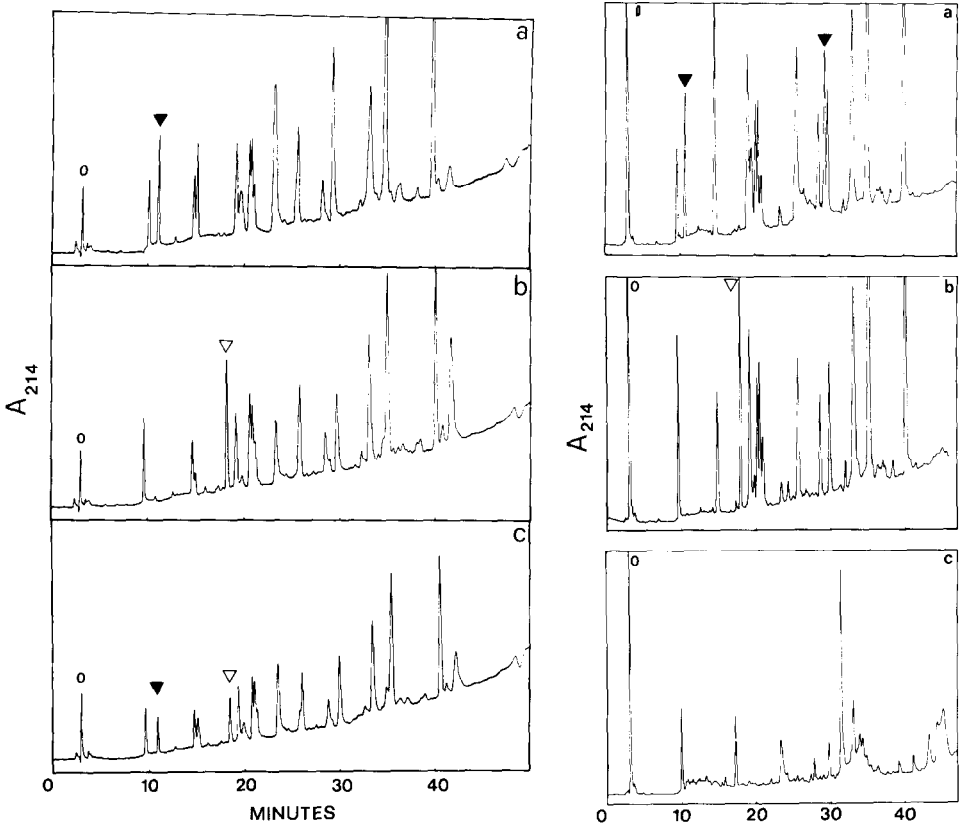

Fig. 2. Liquid chromatographic selected maps of rabbit myosin light chains. Myosin light chains were incubated in 50 mM sodium phosphate buffer (pH 7.8) with *S. aureus* V$_8$ protease (1:500, mol/mol) for 16 h. A 50-min linear gradient from 0 to 50% of acetonitrile in 20 mM potassium phosphate buffer (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 column of μBondapack C$_{18}$ was used. The peak marked 0 is present in all maps and is due to the digestion buffer. Peptides indicated by ▼ are unique to the LC1F map and that indicated by ▽ is unique to the LC3F map. (a) LC1F; (b) LC3F; (c) LC1F + LC3F.

Fig. 3. Liquid chromatographic selected maps of rabbit myosin light chains. Myosin light chains were incubated in 20 mM Tris–acetate buffer (pH 8.1) with *S. aureus* V$_8$ 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 were injected. The flow-rate was 1.0 ml/min. The peak marked 0 is present in all maps and is due to the digestion buffer. Peptides by ▼ are unique to the LC1F map and that indicated by ▽ is unique to the LC3F map. (a) LC1F; (b) LC3F; (c) LC2F.

detailed inspection of the two profiles revealed some distinct differences. In fact, at least one peak for each of LC1F and LC3F in their maps is unique. To be sure that these peaks are really different in the two peptide maps, we 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 LC1F map whereas that marked with an open triangle is distinctive for the LC3F map. The digestion patterns of the myosin light chains are highly reproducible under fixed experimental conditions (amount of protease, temperature, digestion time and digestion buffer): compare, for example, Fig. 1d with Fig. 2b.

It has been reported[14] that the specificity of S. aureus V_8 protease is buffer dependent. To test this fact we digested myosin light chains also in a Tris–acetate buffer[3]. It is evident that the maps obtained after 16 h of digestion with S. aureus V_8 protease in Tris–acetate buffer are not identical with, although they are very similar to, those resulting when phosphate buffer was used (compare Fig. 3a and b with Fig. 2a and b). Nevertheless, distinct peaks are again apparent in the maps of LC1F and LC3F myosin light chains (Fig. 3a and b); these distinctive peaks are actually unique in the two maps, as revealed by the co-analysis of the two digests (not shown). On the other hand, the digest of LC2F, in Tris–acetate buffer, gives a very different profile (Fig. 3c).

DISCUSSION

S. aureus V_8 protease was previously used for the digestion of myosin light chains as a useful alternative to trypsin and chemical cleavage[15–17], according to the electrophoretic procedure described by Cleveland et al.[18], a technique in which the proteolysis is difficult to control. Many systems are currently used for the analysis of peptides for structural studies of proteins. However, the recovery of peptides from conventional sodium dodecyl sulphate gel electrophoresis is difficult and the yield is very low. On the other hand, gel filtration allows a good recovery of peptides but its resolution is poor and it requires long period of analysis and milligram amounts of samples. Our approach allows us to analyse rapidly, in less than 1 h with a nondestructive technique, the product of a digestion performed under controlled temperature, time of incubation and fixed protein to protease ratios.

Chromatographic eluates are monitored routinely at 214 nm, a wavelength that provides an extremely sensitive approach to detection. Monitoring at 280 nm provides additional useful information regarding peptide composition (tyrosine-containing peptides) without performing further amino acid analysis. Actually, as expected from the sequence, LC3F myosin light chain that contains three tyrosine residues[10] shows a petide map with only three peaks when the chromatogram was obtained by monitoring at 280 nm.

The LC1F rabbit myosin light chain is known to differ from the LC3F in only two respects: it contains an additional 41 residues at its N-terminal end, and the eight N-terminal residues of LC3F contain five amino acid replacements compared with the corresponding sequence of LC1F. Apart from these differences, the amino acid sequences of the two light chains appear to be identical[9]. It is then probable that the additional fragments we found in the map of LC1F have their origin in the so-called "difference peptide" of this particular light chain, while the distinctive peptide observed in the digest of LC3F could derive from the N-terminal portion of the mol-

ecule. It is also known from sequence studies[10] that the primary structure of LC2F rabbit myosin light chain is different from that of the two very homologous LC1F and LC3F light chains. Accordingly, the profile obtained after digestion with *S. aureus* V$_8$ protease is completely different from the maps of both LC1F and LC3F light chains.

The method described here is suitable for generating a discrete number of peptides from a protein under *S. aureus* V$_8$ protease digestion in different buffers and offers the possibility of analysing them rapidly and discriminating small differences in amino acid composition. We are now attempting to extend the applicability of this method to the study of myosin light chain with a still unknown structure. Further, it would be of interest to apply this method also to the analysis of the peptide maps of other myofibrillar proteins.

REFERENCES

1 I. Molnár and Cs. Horváth, *J. Chromatogr.*, 142 (1977) 623.
2 M. J. O'Hare and E. C. Nice, *J. Chromatogr.*, 171 (1979) 209.
3 J. Ozols and F. S. Heinemann, *Biochim. Biophys. Acta*, 704 (1982) 163.
4 L. Dalla Libera, A. Margreth, I. Mussini, C. Cerri and G. Scarlato, *Muscle Nerve*, 1 (1978) 280.
5 U. Carraro, L. Dalla Libera and C. Catani, *J. Muscle Res. Cell Motil.*, 2 (1981) 335.
6 L. Dalla Libera, S. Sartore, S. Pierobon Bormioli and S. Schiaffino, *Biochem. Biophys. Res. Commun.*, 96 (1980) 1662.
7 D. Biral, E. Damiani, P. Volpe, G. Salviati and A. Margreth, *Biochem. J.*, 203 (1982) 529.
8 G. F. Gauthier, S. Lowey and A. W. Hobbs, *Nature (London)*, 274 (1978) 25.
9 G. Franks and A. G. Weeds, *Eur. J. Biochem.*, 44 (1974) 317.
10 J. H. Collins, *Nature (London)*, 258 (1976) 699.
11 D. Biral, L. Dalla Libera, C. Franceschi and A. Margreth, *J. Immunol. Methods*, 31 (1979) 93.
12 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
13 C. R. Merril, D. Goldman, S. A. Sedman and T. Ebert, *Science*, 211 (1981) 1437.
14 J. Houmard and G. R. Drapeau, *Proc. Nat. Acad. Sci. U.S.*, 69 (1972) 3506.
15 L. Reeburgh Keller and C. P. K. Emerson, *Proc. Nat. Acad. Sci. U.S.*, 77 (1980) 1020.
16 S. Ishiura, A. Takagy, I. Nonaka and H. Sugita, *J. Biochem.*, 90 (1981) 279.
17 P. Cummins, *Biochem. J.*, 205 (1982) 195.
18 D. W. Cleveland, S. G. Fischer, M. W. Kirschner and U. K. Laemmli, *J. Biol. Chem.*, 252 (1977) 1102