Journal of Chromatography, 566 (1991) 427–434
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5810

Use of anion-exchange high-performance liquid chromatography for the study of smooth muscle myosin light-chain kinase and its catalytic domain

L. DALLA LIBERA* and M. FASOLO

National Research Council Unit for Muscle Biology and Physiopathology, Institute of General Pathology, University of Padova, Via Trieste 75, 35131 Padova (Italy)

and

P. CAVALLINI, E. RATTI and G. GAVIRAGHI

Glaxo Research Laboratories, Verona (Italy)

ABSTRACT

Avian myosin light-chain kinase from smooth muscle of the gizzard and its catalytic domain, derived from the intact enzyme by trypsin digestion, was purified within 30–40 min by both analytical and preparative anion-exchange high-performance liquid chromatography. The proteins obtained were more than 95% pure and retained their biological activity. The high-performance anion-exchange chromatography protocols represent a significant decrease in purification time when compared with conventional ion-exchange chromatography.

INTRODUCTION

Myosin light-chain kinases (MLCKs) are calcium-calmodulin-dependent enzymes that phosphorylate the 20-kDa regulatory light chain of myosin [1]. MLCKs have been isolated from a variety of sources including smooth muscle, skeletal muscle, cardiac muscle, platelets and macrophages [2]. Whereas in smooth muscle and non-muscle tissues phosphorylation is obligatory for the development of tension, in skeletal muscle it has only a modulatory role in regulating muscle contraction. The molecular mass of the enzyme varies considerably depending on its source, ranging from about 140 kDa for avian gizzard smooth muscle to 75 kDa for mammalian skeletal muscles. The observed differences in the various MLCKs examined must arise from alterations in the primary structure of the proteins. However, all these enzymes are characterized by a similar structure. The MLCKs can be divided into three distinct structural domains: an amino-terminal "tail" of unknown function, a central catalytic core and a carboxy-terminal calmodulin-binding regulatory region [3]. The proteolytic susceptibility of MLCK has been utilized to define the relative location of the catalytic

0378-4347/91/$03.50

(active site) and regulatory (calcium-calmodulin binding site) domains of the enzyme.

Most protocols purify MLCK by conventional open-column chromatographic procedures. However, as the ion-exchange chromatographic separation of MLCK normally requires an extensive fractionation time and is unsuitable for the preparation of the enzyme from small muscles, other approaches such as high-performance anion-exchange chromatography have been evaluated.

It has been shown previously that high-performance anion-exchange chromatography can be successfully used to purify MLCK from complex mixtures [4]. This paper demonstrates that the high-performance liquid chromatographic (HPLC) method is suitable for purifying native MLCK and its catalytic domain from avian smooth muscle. The proposed HPLC procedure can be used to fractionate microgram or milligram amounts of protein using an analytical or preparative porous resin-based anion-exchange column and a linear gradient of sodium chloride solution. The polypeptides can be fractionated to more than 95% purity in 30–40 min.

EXPERIMENTAL

Preparation of avian gizzard extract

The extracts of chicken and turkey gizzard loaded in the analytical HPLC column were prepared according to the procedures described by Dalla Libera *et al.* [4]. The fraction enriched in MLCK by DEAE–Sephacel chromatography and loaded in the preparative HPLC column was prepared as described by Cavallini *et al.* [5].

Tryptic digestion of myosin light-chain kinase

MLCK (390 μg/ml), purified as described by Cavanni *et al.* [6] was incubated at 24°C for 60 min in digest buffer [40 m*M* Tris–HCl (pH 7.6), 20 m*M* NaCl, 1 m*M* dithiothreitol, 1 m*M* ethylene glycol bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA)] with the addition of trypsin (1:100, w/w) to initiate the reaction. The reaction was stopped by the addition of soybean trypsin inhibitor (in a five times weight excess over trypsin) and the peptides were stored at − 20°C.

Assay of myosin light-chain kinase activity

The assay of MLCK activity was carried out as described by Cavanni *et al.* [6]. The extent of phosphorylation was monitored by glycerol–urea–polyacrylamide gel electrophoresis [7].

Sodium dodecyl sulphate gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [8].

Chemicals for high-performance liquid chromatography

The water used for the chromatographic separation was deionized (Milli-Q, Millipore, Bedford, MA, U.S.A.). All other reagents were of analytical-reagent grade. The mobile phase solutions were passed through a 0.45-μm Millipore filter and degassed ultrasonically prior to chromatographic separation.

High-performance liquid chromatography

The Perkin-Elmer HPLC system was a Series 3B liquid chromatograph equipped with a Model LC75 variable-wavelength UV detector set at 280 nm, and a Rheodyne Model 7105 sample injector with a 1-ml sample loop.

The anion-exchange separations were performed on analytical (75 mm × 7.5 mm I.D.) or preparative (150 mm × 21.5 mm I.D.) Bio-Gel DEAE-5-PW columns from Bio-Rad. All the chromatographic runs were carried out at room temperature. The experimental details are given in the figure legends. The peaks were collected manually and analysed by SDS-PAGE and MLCK activity.

RESULTS AND DISCUSSION

Analytical high-performance liquid chromatography of native myosin light-chain kinase

When 900 μg of the magnesium extract of chicken gizzard myofibrils were fractionated on a Bio-Gel DEAE-5-PW column with a linear gradient of increasing sodium chloride concentration, several peaks were observed (Fig. 1). The analysis of the starting material (inset Fig. 1, lane b) and of the chromatographic effluents by SDS-PAGE revealed that the peak marked by an asterisk (inset Fig. 1, lane c) consisted of a protein, the molecular mass of which corresponded to that of MLCK purified by conventional techniques (inset Fig. 1, lane d). The purity of this peak was more than 95%. As the HPLC separation was highly reproducible and performed in less than 30 min, it was possible to chromatograph several aliquots of the magnesium extract and collect the peaks corresponding to MLCK to test whether the protein purified by anion-exchange HPLC had retained its enzymatic activity. It was found that the MLCK purified by this procedure was still characterized by its enzymatic activity, phosphorylating the myosin 20-kDa subunit to an extent of 1 mol of phosphate per mol of light chain and that this phosphorylation was calcium-calmodulin-dependent (results not shown).

To confirm the selectivity of the column for MLCK, 900 μg of the magnesium extract derived from the same tissue (the gizzard) but from a different animal species, *i.e.* the turkey (inset Fig. 2, lane b) were analysed. The HPLC profile obtained for turkey gizzard MLCK was similar to that of chicken gizzard (Fig. 2). The effuents corresponding to several of the peaks were analysed by SDS-PAGE. The peak marked by an asterisk contained MLCK (inset Fig. 2, lane c) which retained its enzymatic activity (results not shown). For comparative purposes, turkey MLCK purified by conventional techniques is shown in lane d.

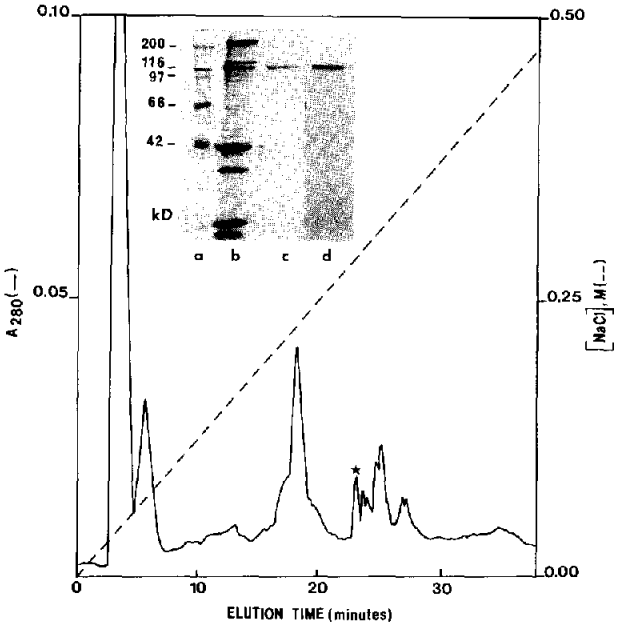

Fig. 1. HPLC profile of magnesium extract derived from chicken gizzard by analytical anion-exchange chromatography. The extract (900 μg) which contained MLCK was chromatographed at 1.0 ml/min on a Bio-Gel DEAE-5-PW analytical column (75 mm × 7.5 mm I.D., Bio-Rad) equilibrated with 100% solvent A using a linear gradient to 100% solvent B in 40 min. Solvent A: 20 m*M* Tris–HCl (pH 8.0). Solvent B: 20 m*M* Tris–HCl (pH 8.0), 0.5 *M* NaCl. Inset: 10% SDS-PAGE profile of purified MLCK. Lane a, molecular mass markers; lane b, starting fraction (600 μl); lane c, protein present in the peak marked by an asterisk; lane d, chicken MLCK purified by standard procedures. The gels were stained with Coomassie blue.

Preparative high-performance liquid chromatography of native myosin light-chain kinase

As analytical HPLC with an anion-exchange column gave good separation of MLCK, it was decided to use this technique on a preparative scale. An anion-exchange preparative column was used to test the possibility of obtaining larger amounts of pure MLCK. Using the same experimental conditions as for the analytical column, acceptable chromatograms could not be obtained. To optimize the separation of MLCK two parameters were changed. First, the starting material was not the crude magnesium extract, but a fraction obtained from the magnesium extract after a pre-purification step by conventional open-column anion-exchange chromatography (DEAE–Sephacel). Second, the elution was achieved with a sodium chloride gradient in 20 m*M* phosphate buffer (pH 8.0). The results are illustrated in Fig. 3 for 5 mg of extract. As the starting material contained fewer components than in the analytical runs (inset Fig. 3, lane b), fewer peaks were observed in the chromatogram. SDS-PAGE demonstrated that it is possible to obtain a reasonably pure MLCK preparation (about 300 μg), with

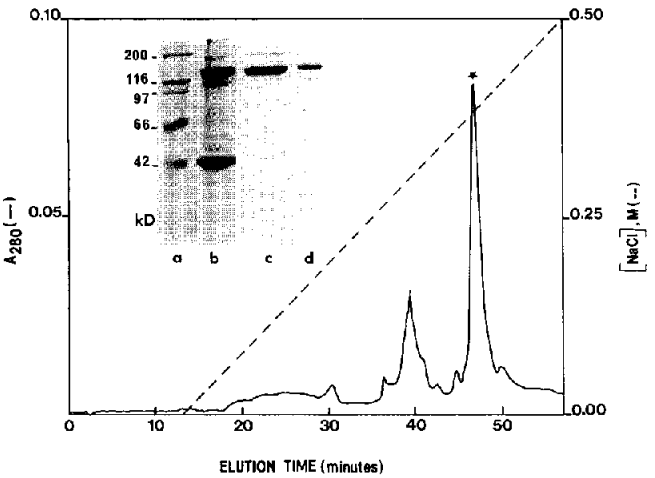

Fig. 2. HPLC profile of magnesium extract derived from turkey gizzard by analytical anion-exchange chromatography. HPLC procedure as in Fig. 1. Inset: 10% SDS-PAGE profile of purified MLCK. Lane a, molecular mass markers; lane b, starting fraction (500 μl); lane c, protein present in the peak marked by an asterisk; lane d, turkey MLCK purified by standards procedures. The gels were stained with Coomassie blue.

the preparative column, slightly contaminated by a 200-kDa component (inset Fig. 3, lane c). Between 5 and 10 mg could usually be chromatographed in one run.

Analytical high-performance liquid chromatography of catalytic domain of myosin light-chain kinase

The proteolysis by trypsin of gizzard MLCK has a biphasic effect on kinase activity. During the initial phase of digestion, calcium-calmodulin-dependent kinase activity is dramatically reduced. Further proteolysis causes an increase in activity which is independent of calcium-calmodulin. A loss of activity is associated with the formation of a 64-kDa fragment, whereas calcium-calmodulin activity is associated with the formation of a 61-kDa fragment, as demonstrated by SDS-PAGE experiments.

To study more precisely the domains of MLCK it was necessary to isolate each component after tryptic digestion. The catalytic domain is particularly important and a basic goal of many workers is to obtain the pure peptide in order to perform structural and enzymatic studies. For this purpose the digest of MLCK (390 μg) obtained after 60 min of proteolysis by trypsin was applied to an analytical Bio-Gel DEAE-5 PW column. The elution profile and the electrophoretic pattern of the fractions are shown in Fig. 4. The 61-kDa peptide was more than 90% pure as determined by an overloaded Coomassie blue-stained gel being contaminated by a component of slightly larger molecular mass (inset of Fig. 4, lane c).

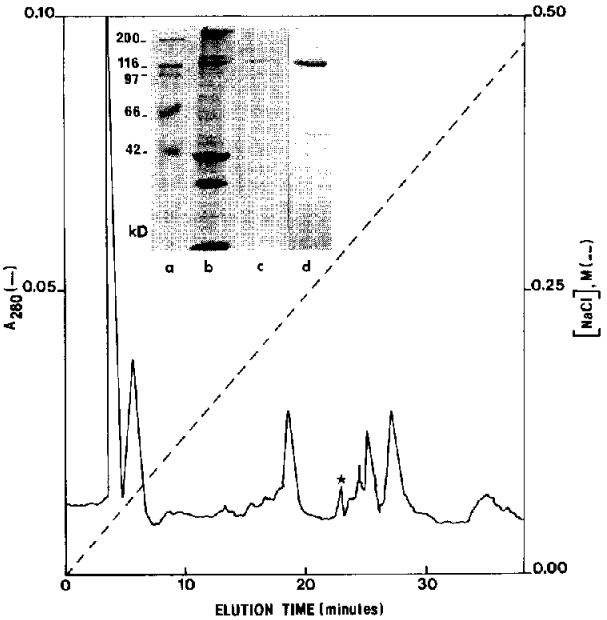

Fig. 3. Preparative HPLC by anion-exchange chromatography of a fraction obtained from chicken magnesium extract after conventional chromatography on DEAE-Sephacel. A 10-ml sample of the fraction which contained MLCK was chromatographed at 4.0 ml/min on a Bio-Gel DEAE-5-PW preparative column (150 mm × 21.5 mm I.D., Bio-Rad) equilibrated with 100% solvent A using a linear gradient to 100% solvent B in 45 min. Solvent A: 20 m*M* sodium phosphate buffer (pH 8.0). Solvent B: 20 m*M* sodium phosphate buffer (pH 8.0), 0.5 *M* NaCl. Inset: 10% SDS-PAGE profile of purified MLCK. Lane a, molecular mass markers; lane b, starting fraction; lane c, protein present in the peak marked by an asterisk; lane d, chicken MLCK purified by standard procedures. The gels were stained with Coomassie blue.

Preparative high-performance liquid chromatography of catalytic domain of myosin light-chain kinase

Unlike native MLCK, the catalytic domain can be purified by preparative HPLC without any change in the chromatographic conditions. In addition, even better results could be obtained with the preparative anion-exchange HPLC column as shown in Fig. 5. The peptide obtained is extremely pure, as revealed by a SDS polyacrylamide gel stained with the highly sensitive silver technique (inset of

TABLE I

PURIFICATION OF CHICKEN GIZZARD MYOSIN LIGHT-CHAIN KINASE

Method	Purity (%)	Enzymatic activity	Purification time (days)
HPLC	95	Retained	1
Conventional	85	Retained	10

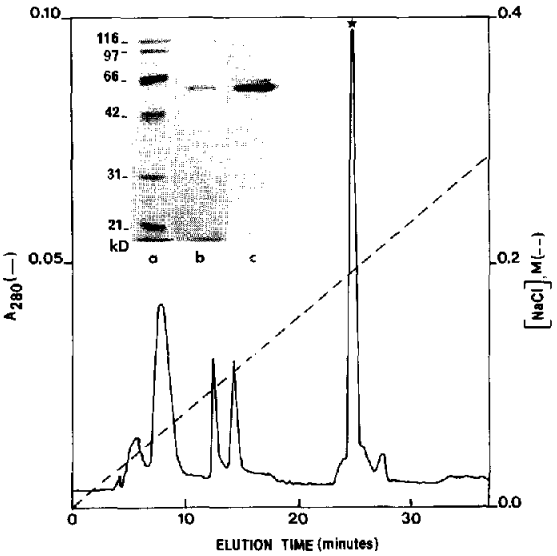

Fig. 4. Separation of tryptic fragments of chicken MLCK by high-performance anion-exchange liquid chromatography. MLCK (390 μg/ml) was proteolyzed with trypsin for 60 min. The digest (390 μg) was injected onto a Bio-Gel DEAE-5-PW analytical column (75 mm × 7.5 mm I.D.) and washed with 30 m*M* Tris–HCl (pH 7.5), 0.1 m*M* dithiothreitol. A 50-min NaCl gradient from 0 to 0.4 *M* was applied at a flow-rate of 0.8 ml/min. Inset: 10% SDS-PAGE profile of purified MLCK. Lane a, molecular mass markers; lane b, starting fraction; lane c, protein present in the peak marked by an asterisk. The gels were stained with Coomassie blue.

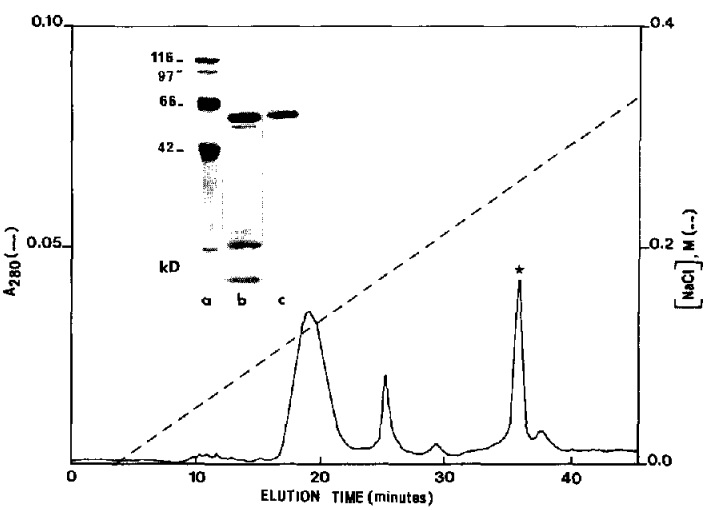

Fig. 5. Separation of tryptic fragments of chicken MLCK by high-performance anion-exchange liquid chromatography. MLCK (210 μg/ml) was proteolyzed with trypsin for 60 min. The digest (800 μg) was injected onto a Bio-Gel DEAE-5-PW preparative column (150 mm × 21.5 mm I.D., Bio-Rad) and washed for a few minutes with 30 m*M* Tris–HCl pH 7.5, 0.1 m*M* dithiothreitol. A 50-min NaCl gradient from 0 to 0.4 *M* was applied at a flow-rate of 4.0 ml/min. Inset: 10% SDS-PAGE profile of purified MLCK. Lane a, molecular mass markers; lane b, starting fraction; lane c protein present in the peak marked by an asterisk. The gels were stained using the silver technique.

Fig. 5, lane c) and there is virtually no trace of the contaminant as with analytical HPLC (see, for comparison, inset of Fig. 4, lane c). The kinase activity of the isolated peptide was determined using glycerol–urea–PAGE. The peptide purified by this procedure is still characterized by its enzymatic activity, phosphorylating the myosin 20-kDa subunit both in the presence and in the absence of calcium (results not shown).

CONCLUSIONS

The HPLC protocols described here provide techniques for fractionating MLCK from crude extracts which have the advantage of increased speed and reproducibility compared to other ion-exchange techniques (see Table I). These purification protocols will be useful for examining the structure of MLCK and the role of its domains.

ACKNOWLEDGEMENTS

We thank the slaughterhouse "Sival-Carni" for providing chicken and turkey gizzards and Mr. S. Belluco for skilful technical assistance. This research was supported in part by a grant form Glaxo SpA (Verona, Italy). Dr. M. Fasolo is the recipient of a fellowship from Glaxo.

REFERENCES

1 R. S. Adelstein and E. Eisenberg, *Annu. Rev. Biochem.*, 49 (1980) 921.
2 M. P. Walsh and D. J. Hartshorne, in W. Y. Cheung (Editor), *Calcium and Cell Function*, Vol. I, Academic Press, New York, 1982, p. 223.
3 H. L. Foyt, V. Guerriero and A. R. Means, *J. Biol. Chem.*, 260 (1985) 7765.
4 L. Dalla Libera, P. Cavallini, M. Fasolo, P. Cavanni, E. Ratti and G. Gaviraghi, *Biochem. Biophys. Res. Commun.*, 167 (1990) 1249.
5 P. Cavalini, P. Cavanni, E. Ratti, G. Gaviraghi and L. Dalla Libera, in U. Carraro (Editor), *Sarcomeric and Non-sarcomeric Muscles: Basic and Applied Research Prospects for the 90's*, Unipress, Padova, 1988, p. 677.
6 P. Cavanni, P. Cavallini, E. Ratti, G. Gaviraghi and L. Dalla Libera, *Biochem. Biophys. Res. Commun.*, 160 (1989) 174.
7 W. T. Perry and S. V. Perry, *Biochem. J.*, 119 (1970) 31.
8 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.