FRACTIONATION OF RABBIT VENTRICULAR MYOSINS BY AFFINITY CHROMATOGRAPHY WITH INSOLUBILIZED ANTIMYOSIN ANTIBODIES

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1. Introduction

Immunofluorescence studies with specific antimyosin antibodies have demonstrated myosin polymorphism in avian [1] and mammalian heart (in preparation). In addition to the existence of distinct types of myosin in atrial and ventricular tissue, myosin heterogeneity has been established in muscle cells of both tissues. For instance, when fluorescein-labelled anti-beef atrial myosin antibodies were applied to sections of the rabbit heart, ventricular muscle cells with varying degree of reactivity were seen, suggesting the presence of multiple forms of ventricular myosin. Electrophoretic evidence for the existence of different myosin isoenzymes in the rat ventricles has been reported [2,3]. We show here that an immunoadsorbent made of specific antibodies against beef atrial myosin permits the fractionation of two myosin populations from rabbit ventricles with the same light chain composition but different heavy chain structure.

2. Experimental

2.1. *Materials*

Rabbit ventricular myosin was prepared as in [4]. Pepstatin A (Sigma) 20 μg/l was added throughout the preparation [5]. Contaminants were removed by anion exchange chromatography on DEAE–Sephadex A-50 (Pharmacia) [6].

Abbreviations: PBS, phosphate buffered saline; GdnHCl, guanidine hydrochloride; SDS, sodium dodecyl sulphate; DTT, dithiothreitol

2.2. *Preparation of immunoadsorbent*

Specific antibodies against atrial beef myosin were raised in rabbits and purified by affinity chromatography (submitted). Specific IgG (13.9 mg) were coupled to 5 g CNBr-activated Sepharose 4B (Pharmacia) following the instructions of the manufacturer. Free reactive CNBr-groups were saturated by 0.1 M glycine in sodium bicarbonate buffer (pH 8.0) for 1 h. The slurry was finally washed with 0.3 M KCl, 50 mM Tris–HCl (pH 7.5) and stored with NaN_3 in refrigerator at 4°C for several months.

2.3. *Immunofluorescence of antimyosin antibodies*

Direct immunofluorescence was performed on cryostat sections using specific anti-beef atrial myosin antibodies coupled with fluorescein isothiocyanate [1].

2.4. *Affinity chromatography of ventricular myosin*

The maximum binding capacity of the column was assayed by measuring the amount of atrial myosin able to bind to the immunoadsorbent (6 mg). An equal amount of column-purified rabbit ventricular myosin in 0.3 M KCl, 50 mM Tris–HCl (pH 7.5) was mixed and gently shaken for 16 h at 4°C with the immunoadsorbent. The slurry was loaded into a 0.9 × 15 cm column (Pharmacia) and the effluent was monitored at 280 nm. After the elution of unretained material the immunoadsorbent was washed with buffer (pH 7.5) until the recorder trace returned to the baseline. Bound material was eluted by single step of 4 M GdnHCl in column buffer [7]. The unbound material was precipitated with 50% $(NH_4)_2SO_4$ and dialyzed against 0.5 M KCl, 0.1 mM

DTT, 10 mM Tris–HCl (pH 7.5). The bound fraction was first exhaustively dialyzed to remove GdnHCl, until no visible reaction could be detected by Nessler reagent, and then treated as the unbound fraction.

2.5. *Two-dimensional gel electrophoresis*

Two-dimensional gel electrophoresis, where the first dimension was isoelectric focusing and the second dimension was SDS–gel electrophoresis, was as in [8], except that a polyacrylamide gradient (5–22%) was used in the second dimension. Slabs were stained with 0.25% Coomassie blue in 50% methanol and 10% acetic acid for 1–2 h at 60°C, and destained in 40% methanol at the same temperature.

2.6. *Tryptic digestion of myosin*

Tryptic digestion of myosin was performed according to [9]. The fragments were analyzed by two-dimensional gel electrophoresis as in section 2.5.

3. Results

3.1. *Immunological heterogeneity of ventricular myosin*

When rabbit ventricular myosin was tested against anti-beef atrial myosin, by double immunodiffusion assay, a weak precipitin band was observed. Figure 1 shows the immunofluorescence staining pattern obtained with the same antibody applied to cryostat sections of rabbit ventricle. It is evident the wide degree of myosin heterogeneity present in ventricular myocardium ranging from completely unreactive fibers to strongly positive fibers with several degrees of intermediate staining.

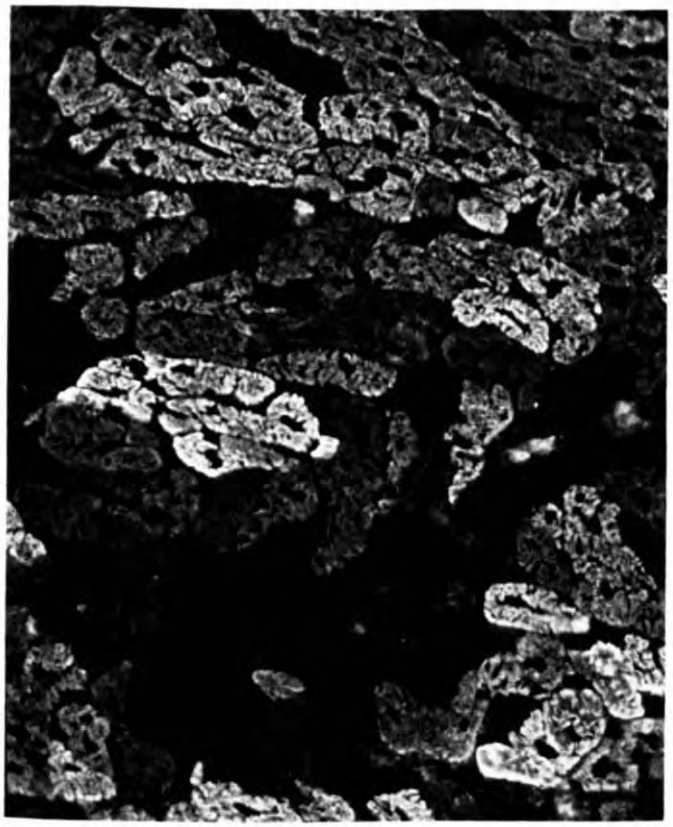

Fig.1. Heterogeneity of rabbit ventricular cells. Cryostat section (~10 μm) was incubated with fluorescein-labelled anti-beef atrial myosin antibodies for 30 min at 37°C, washed with PBS, fixed in 1.5% paraformaldehyde in PBS and mounted in Elvanol. The fluorescence was observed in a Leitz Ortholux microscope equipped with epi-illumination apparatus.

3.2. *Immunochemical fractionation of ventricular myosin*

When ventricular myosin was applied to a column of insolubilized anti-beef atrial antibodies, only a proportion of myosin was retained. A typical elution profile is shown in fig.2. The first peak (Va myosin) represents unbound myosin which is unretained by the immunoadsorbent. The second peak (Vb myosin) remained bound to the specific anti-beef atrial myosin antibodies and was eluted with 4 M GdnHCl. The ratio of unbound:bound protein was about 3:1. To test that no Vb myosin was present in the unretained fraction, the two myosins isolated by affinity chromatography were assayed by double immunodiffusion against specific anti-beef atrial myosin IgG [1] and only Vb displayed a precipitin band.

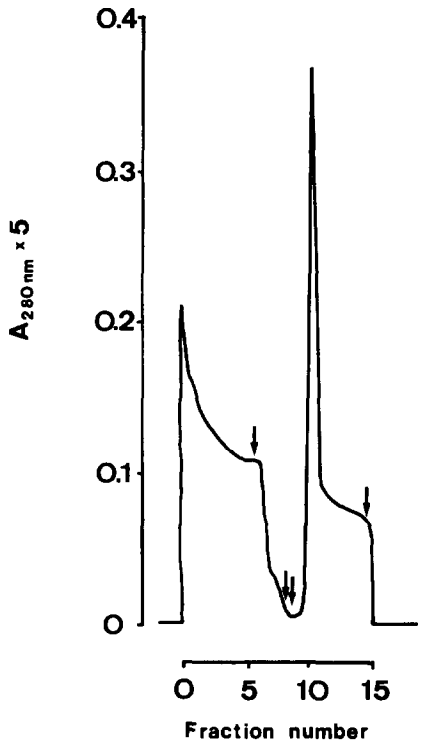

Fig.2. Elution profile of ventricular myosins. The first peak represents unbound myosin (Va) and the second peak bound myosin (Vb). Single arrow indicates the washings with column buffer; double arrows indicate the addition of 4 M GdnHCl. The fraction volume was ~2 ml. This profile is representative of 10 separate experiments.

3.3. *Two-dimensional analysis of myosin light chains*

Analysis of light subunit composition of unfractionated, Va and Vb myosin by two-dimensional gel electrophoresis is shown in fig.3. It is evident that all the myosins subjected to this analysis display identical light chain compositions regarding molecular weight and isoelectric point. Two light chains, LC1 (27 000 mol. wt) and LC2 (20 000 mol. wt) can be recognized; the minor spot just on the right of LC2 is probably the phosphorylated form of this light chain [10].

3.4. *Two-dimensional tryptic peptide maps of ventricular myosins*

Figure 4 shows the two-dimensional tryptic peptide map of unfractionated, Va and Vb myosin and the coelectrophoresis of Va and Vb. Comparison of the maps of Va and Vb (fig.4b,c) revealed that $\geqslant 5$ peptides were typical of Va and not present in Vb; on the other hand 3 peptides are typical of Vb map. Moreover, there are some peptides which are present in different relative amounts in both maps. Since we have used an equimolecular mixture of the two digests, the coelectrophoresis of Va and Vb (fig.4d) partially resembles the map of unfractionated myosin (fig.4a) where the two populations are naturally present in 3:1 ratio.

4. Discussion

Multiple forms of myosin were identified in rat ventricular myocardium by electrophoresis of native molecular [2,3]. This study shows that two myosin subpopulations can be fractionated by affinity chromatography with insolubilized specific anti-myosin antibodies. The two forms of myosin were found to differ in heavy chain structure but possess identical light chain pattern. As demonstrated by immunofluorescence staining these two myosins appear distributed in different amounts in ventricular cells. The intermediate degree of staining of some ventricular cells with fluoresceinated antibodies, could be due to the presence of a mixture of the two myosin populations or, to an hybrid form of the two molecules. The presence of hybrid forms of myosin was suggested by the finding of a third myosin component in rat ventric [3]. Our peptide maps, con-

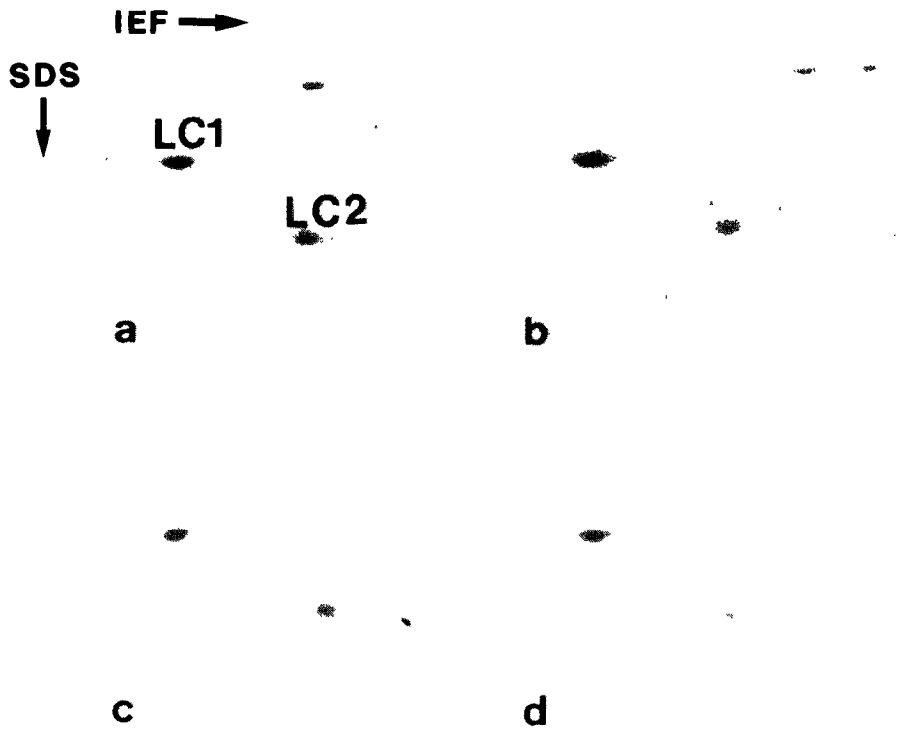

Fig.3. Two-dimensional gel electrophoresis of myosin light chains. Protein was ~40 μg/slab; in (d) 20 μg of each myosin were used. Only the light chain area is represented in the figures. (a) Unfractionated myosin; unbound myosin (Va); (c) bound myosin (Vb); (d) b+c.

cerning essentially the globular heads of the molecule, show that a few peptides are common to the two ventricular myosin forms; however other peptides are unique to each form, a finding not easily compatible with the presence of hybrid forms, which should be retained by the immunoadsorbent. The few common peptides may rather represent regions of limited homology in the sequence of the heavy chains of the two myosins.

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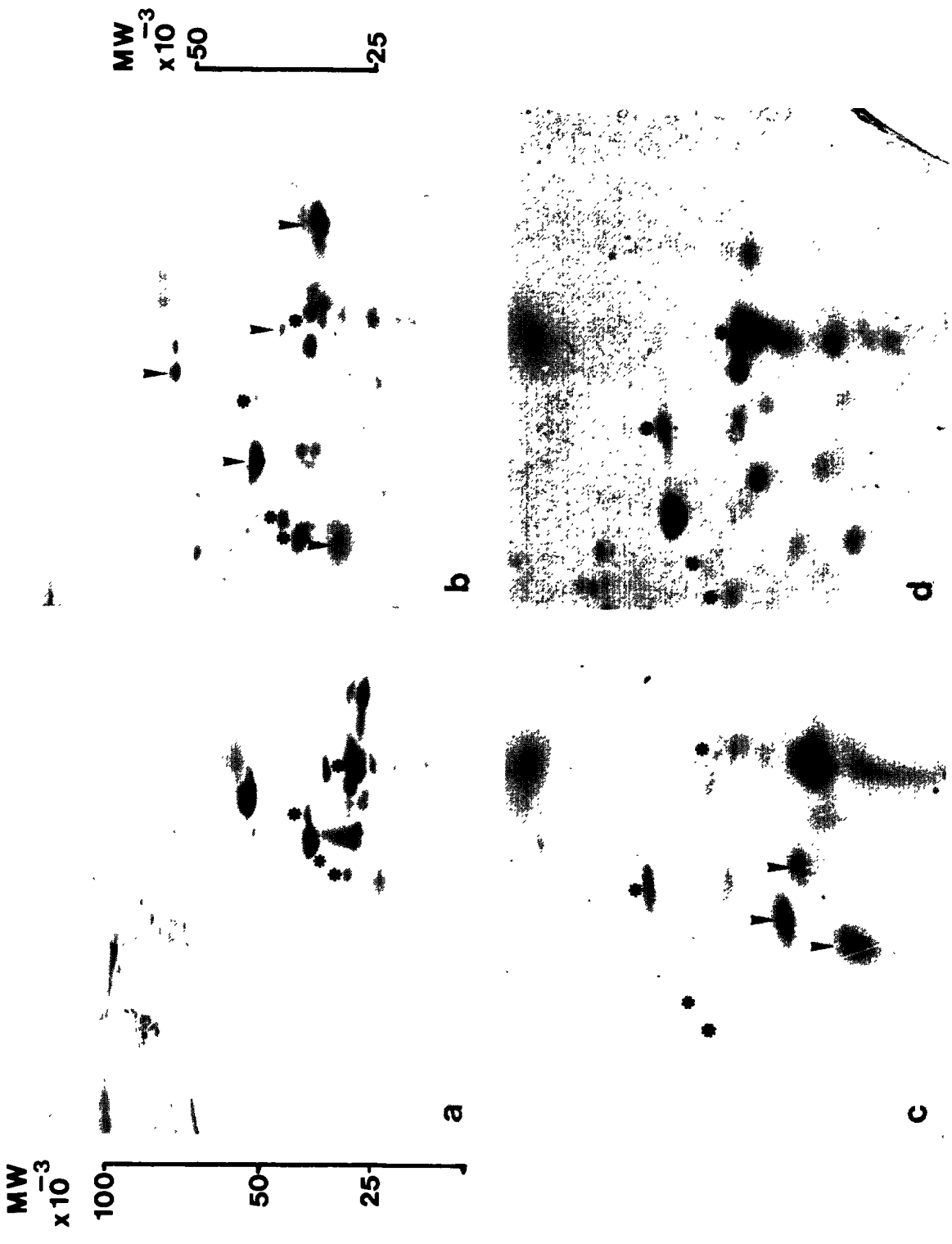

Fig.4. Two-dimensional gel electrophoresis of low ionic strength soluble tryptic peptides of ventricular myosins. Protein was ~300 μg and in (d) ~150 μg of each digest. Vertical arrowheads indicate peptides typical of Va and Vb. The peptides labeled with an asterisk are common to all myosins. In (b–d) only the lower right area of the slab is shown magnified ~2 times.