Small and Large Scale Preparative Purification of Myosin Light and Heavy Chains by Selective KDS Precipitation of Myosin Subunits: Yield by SDS PAGE and Quantitative Orthogonal Densitometry.

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Abstract

A new simple method to purify Myosin Light Chains (MLC) and Myosin Heavy Chains (MHC) by means of KCl selective precipitation of Sodium Dodecyl Sulfate (SDS)-denatured myosin subunits is described. Studying the role of temperature, pH, SDS and solutes concentrations on Potassium Dodecyl Sulfate (KDS)-protein precipitation we previously recognised that changes in these parameters allow selective repartition of desired proteins in pellets or supernatants (Carraro et al. 1991. *Electrophoresis* 12: 1005-1010). A two-step procedure based on pH differential solubility of KDS-myosin subunits is described for the sequential precipitation of MHC and MLC. Quantitative analysis by orthogonal densitometry of Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) slabs shows that almost pure MHC are recovered after step one. The purity of MLC strongly depends on the purity of myosin, since KDS effectively concentrate contaminants. Though assembled from established procedures, for the logic extensions introduced, the new procedure allows to easily obtain MHC or MLC also from highly diluted myosin solutions. Indeed it is a powerful, unique tool if muscle biopsies or *in vitro* cultures ought to be handled.

Key words: MHC, MLC, KDS-protein precipitation, SDS PAGE, Quantitative orthogonal densitometry.

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Myosin is an hexapolypeptide made of two heavy chains and four light chains, isoforms of which are segregated in different fiber types and/or developmental stages [20]. Myosin heavy chains (MHC) are polypeptides of MW of about 200,000 Daltons and their isoforms can be separated by SDS 6% PAGE [6]. At gene level at least six sarcomeric MHC have been recognised: α-Cardiac, β-Cardiac or MHC1, MHC2A, MHC2B, MHCEmb, MHCNeo [3].

Myosin Light Chains (MLC) are polypeptides of MW of about 20,000 Daltons, which also exist in several isoforms. The heterogeneity of light chains is easily detectable by one- and two-dimensional gel electrophoresis [3, 7]. Cromatography is the reference method for preparative separation of proteins which differ in molecular weight but procedures are laborious and time consuming or, if brief as in the case of HPLC, they need expensive instrumentation to purify minute amounts of proteins. Another approach is Electroendosmotic Preparative Gel Electrophoresis (EPGE) which allows to collect from complex mixtures of crude myosins or from tissue homogenates MLC and MHC isoforms in almost pure form [22].

Precipitation, on the basis of solubility in an aqueous solution of salts and organic solvents, is one of the better established methods for protein recovery, but often the low concentration of required solutes does not permit this approach, in particular if denatured with ionic detergents as in the EPGE case [13]. Recently we overcame this problem [8]; now we are applying this result to develop a new preparative procedure of MLC and MHC which requires hours of bench work and no instrumentation investments. Selective KDS-protein precipitation is a new two-step method which allows to obtain from purified myosin almost pure MHC and enriched MLC fractions. This simple, efficient and inexpensive technique permits also to fractionate and collect proteins from highly diluted myosin solutions.

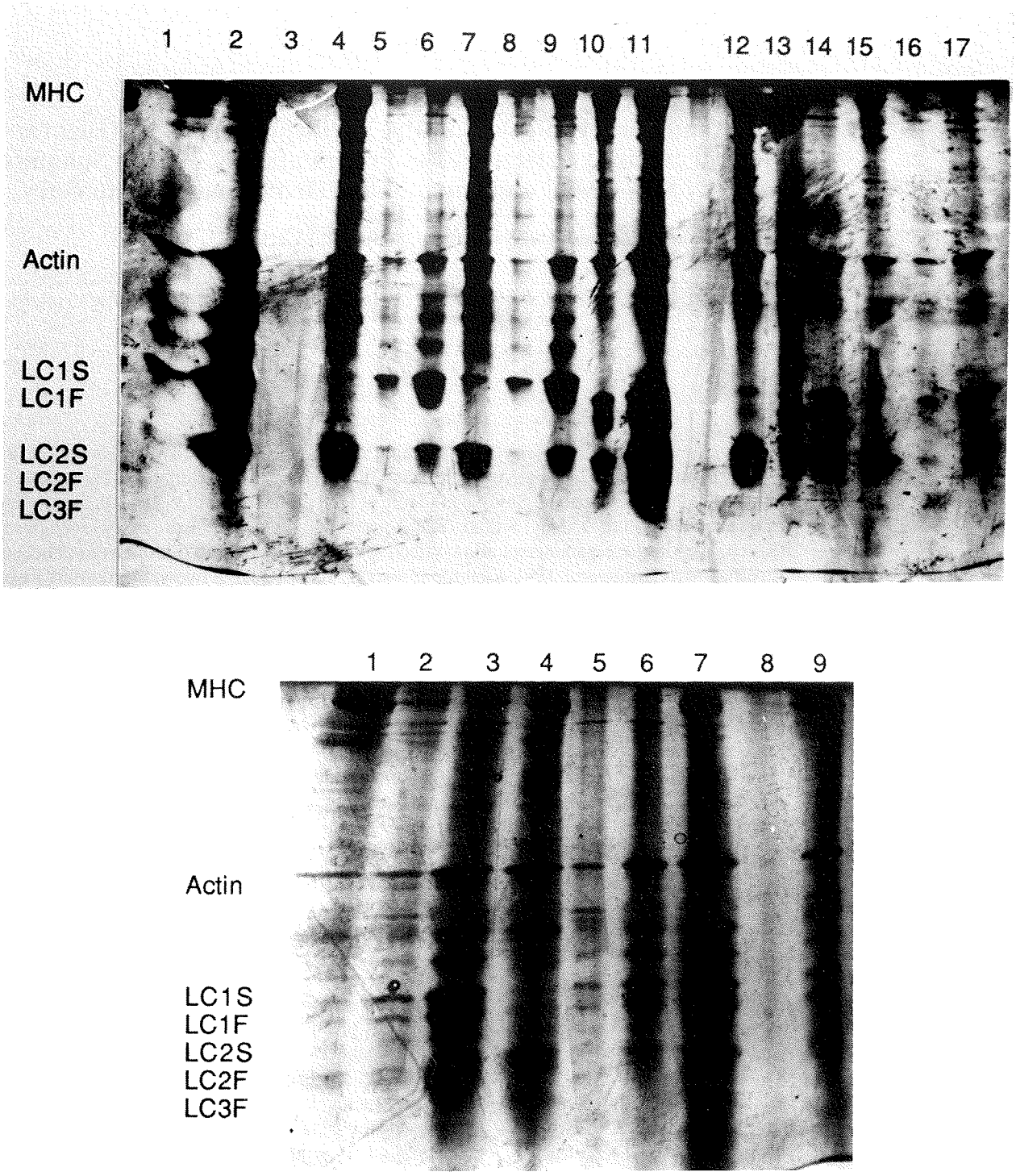

Figure 1. Effects of pH on differential precipitation by 180 mM KCl (final concentration) of SDS-solubilized myosin subunits. SDS 12% PAGE followed by silver stain.

A, Lane 1: rat soleus myosin 2.5 µg in 0.2 % SDS, 25 mM Tris, 192 mM glycine, pH 8.3 (Sol. G) (sample before KCl); lane 2: KDS precipitate and lane 3: supernatant of soleus myosin in Sol. G treated with TCA to pH 1 and KCl to 180 mM; lane 4: KDS precipitate and lane 5: supernatant of soleus myosin in Sol. G treated with KCl to 180 mM; lane 6: KDS precipitate of sample analysed in lane 5 after treatment with TCA to pH 1 and KCl to 180 mM; lane 7: KDS precipitate and lane 8: supernatant of soleus myosin in Sol. G treated with 1 N NaOH to pH 10 and KCl to 180 mM; lane 9: KDS precipitate of sample analysed in lane 8 treated with TCA to pH 1 and KCl to 180 mM. Lanes 10: Rat EDL myosin, 2.5 µg, in Sol G (sample before KCl addition); lane 11: KDS precipitate and lane 12: supernatant of EDL myosin in Sol. G treated with TCA to pH 1 and KCl to 180 mM; lane 13: KDS precipitate

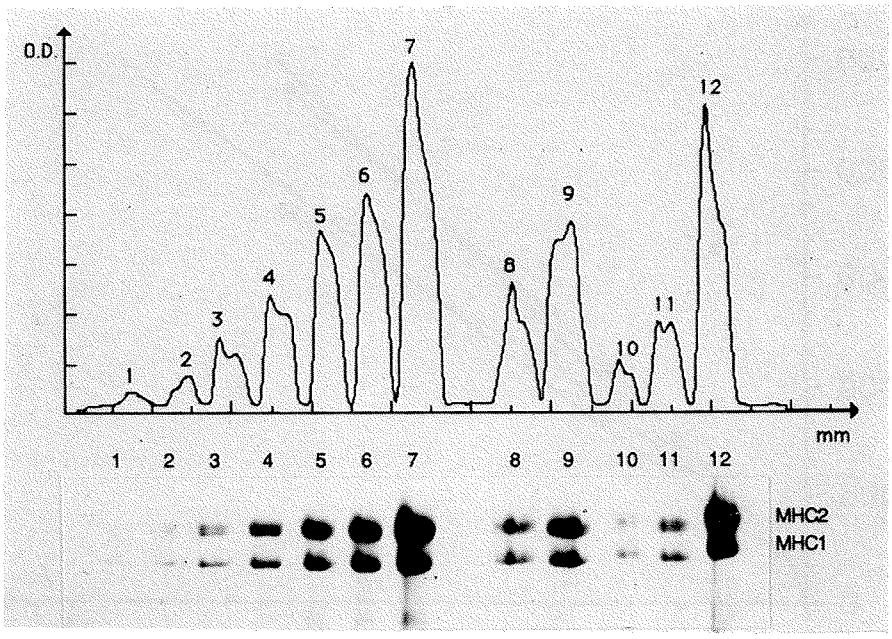

Figure 2. MHC recovered after step-one and step-two KDS-precipitation. Lanes 1-7: mixtures of myosin markers from soleus and EDL rat muscles 0.5, 1, 2.5, 5, 7.5, 10, 20 µg, respectively. Lanes 8, 9 and 11: step-one KDS-MHC precipitation from 5 ml of 50 µg/ml EDL and soleus rat myosins at pH 1, 8 and 10, respectively. Lanes 10 and 12: step-two KDS-MHC precipitation from supernatants of the pellets shown in lanes 9 and 11, but loaded in a ten-time larger volumes than the samples of lanes 8, 9 and 11. Inset: Orthogonal densitometry of MHC1 bands in Figure 2. Peaks 1-7: mixtures of myosin markers from soleus and EDL rat muscles 0.5, 1, 2.5, 5, 7.5, 10, 20 µg, respectively. Peaks 8, 9 and 11: step-one KDS-MHC precipitation from 5 ml of 50 µg/ml EDL and soleus rat myosins at pH 1, 8 and 10, respectively. Peaks 10 and 12: step-two KDS-MHC precipitation from supernatants of pellets shown in lanes 9 and 11, but loaded in a ten-time larger volumes than the samples of lanes 8, 9 and 11. Note the spectacularly flat baseline and the reliable reproduction of bands irregularities.

Materials and Methods

Myosin extraction

Myosin was purified from Extensor Digitorum Longus (EDL) and Soleus muscles of adult rats essentially according to Carraro et al. [10], with the following modifications. All procedures were performed at 2-4° C. Muscle tissue was homogenized (Polytron PT 10 OD), using 50 mM KCl containing 10 mM Ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 200 mM Pepstatine, 80 mM Phenilmethilsulfonyl Fluoride (PMSF), 1 mM Benzamidin (Sol. A), to reduce effects of endogenous proteases. After centrifugation at 650 x g for 10 minutes the supernatant was carefully discarded with the floating lipid layer and the pellet washed three-five times with Sol A until the supernatant became clear. The myosin was then extracted for 20 minutes with 0.3 M KCl, 0.15 M K-phosphate buffer, pH 6.5, 10 mM Mg Acetate, 10 mM EGTA, 200 mM Pepstatine, 80 mM PMSF, 1 mM Benzamidin to which 5 mM ATP was added before use

and lane 14: supernatant of EDL myosin in Sol. G treated with KCl to 180 mM; lane 15: KDS precipitate of sample analysed in lane 14 treated with TCA to pH 1 and KCl to 180 mM; lane 16: KDS precipitate and lane 17: supernatant of EDL myosin in Sol. G treated with 1 N NaOH to pH 10 and KCl to 180 mM; lane 18: KDS precipitate of sample analysed in lane 17 treated with TCA to pH 1 and KCl to 180 mM.

B, Lane 1: KDS precipitate and lane 2: supernatant of EDL and soleus co-electrophoresis in Sol. G treated with 1 N NaOH to pH 10 and KCl to 180 mM; lane 3: KDS precipitate of sample analysed in lane 2 treated with TCA to pH 1 and KCl to 180 mM; lane 4: KDS precipitate and lane 5: supernatant of EDL and soleus co-electrophoresis in Sol. G treated with KCl to 180 mM; lane 6: KDS precipitate of sample analysed in lane 5 treated with TCA to pH 1 and KCl to 180 mM; lane 7: KDS precipitate and lane 8: supernatant of EDL and soleus co-electrophoresis in Sol. G treated with TCA to pH 1 and KCl to 180 mM. Lane 9: mixture of rat EDL and soleus myosins, 2.5 µg, in Sol. G (sample before KCl addition).

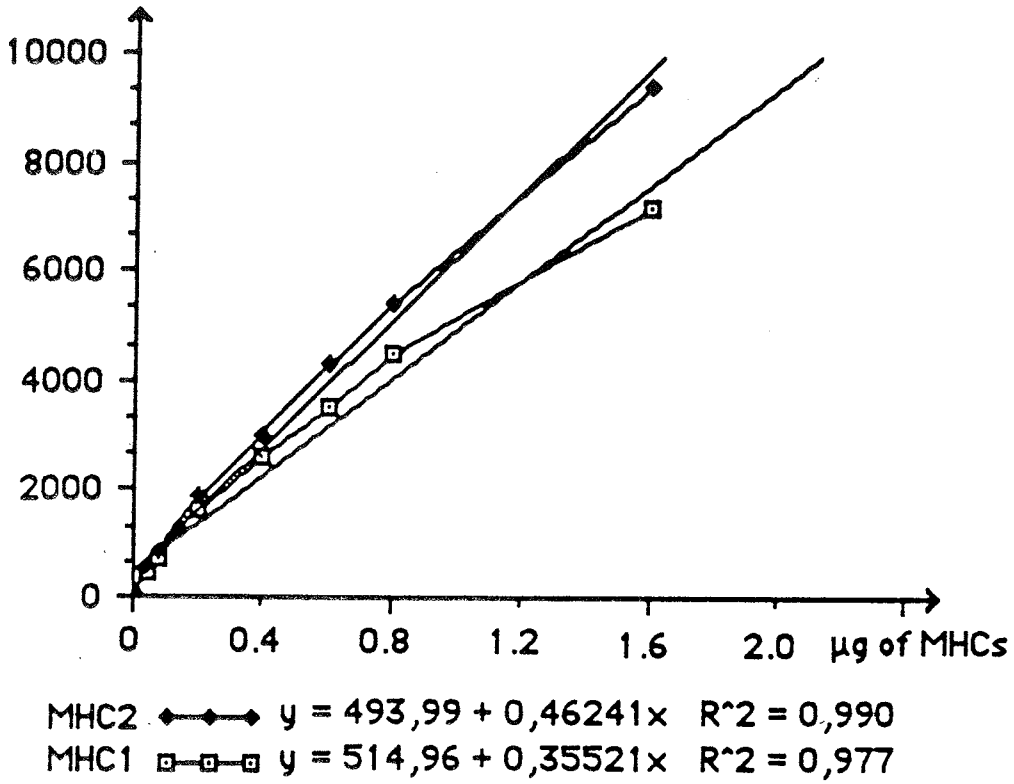

Figure 3. Correlation between protein load and densitometric peak areas.

(Sol. B). Approximately 25 ml of Sol. B was used per gram of fresh muscle. After 30 minutes of mild agitation samples were centrifuged at 20,000 x g for 20 minutes and then the supernatant at 150,000 x g for two hours. The supernatant was dialyzed for 4 hours or overnight in 10 mM Tris-HCl pH 7.2, 50 mM KCl, 0.1 mM dithiothreitol, 5 mM EGTA (Sol. C). Myosin was collected by centrifugation at low speed, dissolved in 0.5 M NaCl, 50 % v:v glycerol (Sol. D) by addition of 1 M NaCl and glycerol and stored at -20° C. When myosin was needed it was precipitated by dilution at low ionic strength with cold water and centrifugation at 650 x g for 10 minutes at 4° C.

One dimensional analysis of MLC

Analysis of MLC was performed in SDS 12% PAGE essentially according to Laemmli [18] but 20% v/v glycerol was present in the separating gel [8].

One dimensional analysis of MHC

Analytical SDS PAGE of MHC was performed on 6% polyacrylamide slabs 0.75 mm x 130 mm x 130 mm [6]. Slabs were prepared according to Laemmli [18], but 37.5% v/v glycerol was present in separating and stacking gels [12]. Electrophoretic buffer was: 25 mM Tris, 192 mM glycine pH 8.3, 0.1% SDS (Sol. E). Overnight separation of MHC was achieved using constant current mode at about 6 mA per slab, but tuning the value to that which gave an initial voltage of 40 Volts. Usually gel electrophoresis started late in the afternoon and the front dye ran out of the slab during the night. After 16-18 hours of electrophoresis the voltage rose to 180-200 Volts and the run was stopped. When the majority of the bands in a slab contained more than 0.2 µg of protein, the slab was stained with 0.1% Coomassie blue in 5% Acetic Acid, 40% Ethanol. If bands contained less than 0.1 µg of protein the slab was stained with the silver method of Merril et al. 1981 [19] as modified by Betto 1989 [2].

Quantitative orthogonal densitometry

Densitometric scanning was performed using a GS-300 Transmittance-Reflectance Scanning Densitometer (Hoefer Scientific Instruments) connected to a Macintosh Plus or SE (Apple Computer). Data were processed using the GS-370 Data System for the Hoefer GS-300 Scanning Densitometer, Macintosh version. Slabs were scanned first along the electrophoretic direction and then along the axis perpendicular to the electrophoretic migration. Densitometric values are linear between 0.25-10 µg of protein.

Standard procedure of KDS-protein precipitation

After studying factors which influence precipitation and recovery of SDS-proteins, the following standard procedure of KDS-protein precipitation was defined to obtain an effective recovery of highly diluted proteins [8]. Native proteins: To a dilute solution of proteins an equal volume of 0.2% SDS, 25 mM Tris, 192 mM glycine, pH 8.3, is added, followed by either boiling for 3 min or incubating for 30 min on ice. Proteins already solubilized with SDS:

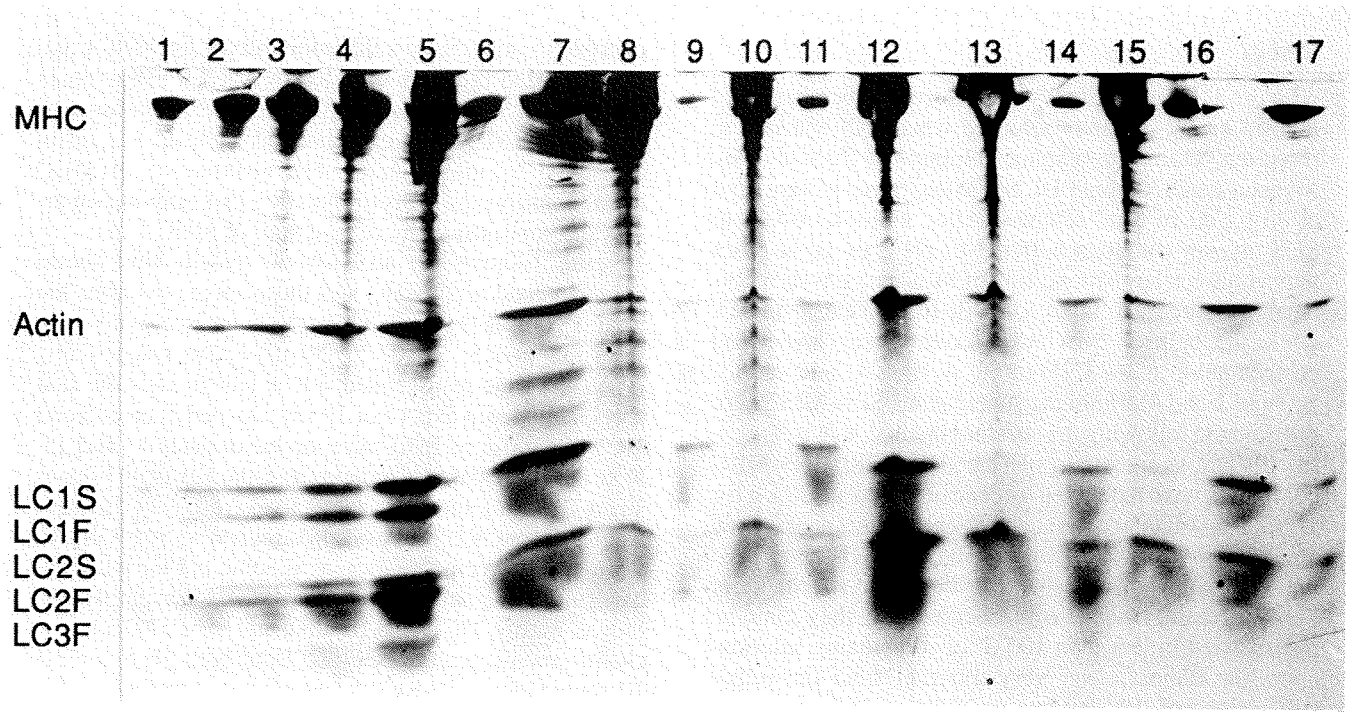

Figure 4. Step-one and step-two KDS-precipitation of myosin light chains. Lane 1-5: Mixtures of myosin markers from EDL and soleus rat muscles 2.5, 5, 10, 20, 50 µg, respectively. Lane 6: rat soleus myosin, 2.5 µg, in Sol. G (sample before KCl addition); lane 7: KDS precipitate of soleus myosin in Sol. G treated with TCA to pH 1 and KCl to 180 mM; lanes 8 and 10: step-one KDS-precipitated proteins at pH 8 and 10, respectively; lanes 9 and 11: step-two KDS-precipitated proteins of samples analysed in lane 8 and 10, respectively. Lane 12: KDS precipitate of EDL myosin in Sol. G treated with TCA to pH 1 and KCl to 180 mM; lanes 13 and 15: step-one KDS-precipitated proteins at pH 8 and 10, respectively; lanes 14 and 16: step-two KDS-precipitated proteins of samples analysed in lane 13 and 15, respectively; Lane 17: rat soleus myosin, 50 µg/ml, in Sol. G (sample before KCl addition).

add 25 mM Tris, 192 mM glycine, pH 8.3 to dilute SDS to a final concentration of 0.1-0.2%. Add ice-cold trichloroacetic acid (TCA) to lower the pH to less than 2.0 (usually to a final concentration of 10% TCA). Keeping the acidified solution on ice, add 2 M KCl to a final concentration of 180 mM. Centrifuge in a conical glass test tubes at 650 x g for 15 min at 2-4° C. Remove the supernatant. If SDS-PAGE had to be performed KDS-protein pellets were resuspended with 10% glycerol, 62.5 mM Tris-HCl, pH 6.8 (Sol. F). Aliquots of the milky KDS-protein suspension were loaded into the SDS PAGE wells. During electrophoresis the dodecyl sulfate ions replace protein-bound KDS, solubilizing the proteins. Alternatively KDS-protein pellets can be solubilized in Sol. F and the SDS-proteins can be renaturated by efficient methods of detergent removal and protein refolding before storage or further analyses [14, 17, 21, 23].

Setting up this standard KDS-Protein precipitation procedure we noticed that some proteins showed a differential sensitivity to KDS-precipitation. Starting from this observation we have established the following selective approach.

Temperature and pH-dependent selective KDS precipitation of myosin subunits

A) Proteins already solubilized with SDS:

1) Add 25 mM Tris, 192 mM glycine, pH 8,3, 0.1% SDS to dilute SDS to a final concentration of 0.2% (Sol. G);

2) At room temperature add NaOH 1N to reach pH 10 (usually to a final concentration of about 0.15 N NaOH);

3) At room temperature add KCl to a final concentration of 180 mM;

4) After fifteen minutes at room temperature, centrifuge in conical glass test tubes at 650 x g for 15 minutes at 2-4° C;

5) Remove the supernatant with a pipette Pasteur;

6) Keep the supernatant on ice for 10 minutes and then add ice cold 100% TCA to lower pH to 1 (usually to a final concentration of 10% TCA);

7) Increase potassium concentration in the supernatant to 180 mM KCl;

8) Centrifuge in conical glass test tubes at 650 x g for 15 minutes at 2-4° C;

9) Remove the supernatant with a pipette Pasteur;

10) If SDS PAGE has to be performed, KDS-Protein pellets are resuspended with 10% glycerol, 62.5 mM Tris-HCl, pH 6.8 (Sol. F). During electrophoresis the dodecyl sulfate ions replace the protein-bound KDS and solubilize the proteins.

Results

Figure 1, A and B shows that selective precipitation of myosin heavy chains is achieved when KCl is added at room temperature to alkaline solutions of SDS-Myosin. Myosins purified from EDL and soleus muscles were used at 50 μg/ml in 5 ml samples. After addition of KCl to SDS-myosin solutions buffered to pH 8 or 10 at room temperature, a large proportion of MLC remains in supernatant (Figure 1, A: lanes 5, 8, 13, 16; B: lanes 2 and 5), while the bulk of MHC were collected by low speed centrifugation at 2-4° C (Figure 1, A: lanes 4, 7, 12 and 15; B: lanes 1 and 4). From the supernatants SDS-MLC were precipitated following the standard method, that is, lowering pH and temperate to pH 1 and 0° C [8]. SDS-MLC complexes were recovered by centrifugation at 650 x g for 15 min in small precipitates which contain a very low amount of free SDS (Figure 1, A: lanes 6, 9, 14 and 17; B: lanes 3 and 6). These step-two pellets were so small that they were hardly visible with the naked eye: they could be resuspended in less than 100 μl of solubilizing solution, appearing almost clear, very different from the milky suspensions of the step-one precipitates. Indeed for the most part SDS (more than 90%) was precipitated with the MHC so that almost only KDS-protein complexes precipitated after step two (Sandri et al. manuscript in preparation).

Figure 2 shows the SDS 6% PAGE analysis of MHC precipitated after step-one (KDS-precipitation at alkaline pH), and those remaining in supernatants and then precipitated at pH 1. Lanes 1-7 show known amounts of a mixture of myosin markers (0.5, 1, 2.5, 5, 7.5, 10, 20 μg, respectively). In lanes 8, 9, 11 the KDS-MHC precipitates at pH 1, 8 and 10, respectively; lanes 10 and 12 contain the resuspended precipitates from pH 8 and 10 supernatants, but loading volumes ten times larger than those of lanes 8, 9 and 11. Note that the bands in lanes 9 and 11 are comparable in size and stain to those in lanes 4 and 5, demonstrating that the step-one precipitates contain more than 90% of the SDS-solubilized myosin heavy chains. Indeed the step-two precipitates in lane 9 and 11 show only traces of MHC, while bands of lanes 10 and 12 are similar in size to those in lanes 6 and 7, demonstrating that, taking into account the loaded volumes, the amount of proteins remaining after precipitation at alkaline pH is less than 10%.

Inset of Figure 2 is the densitometric pattern of MHC1 bands of Figure 2, when PAGE slab was scanned along the axis perpendicular to the electrophoretic migration (orthogonal densitometry). Note that several peaks have an indented profile, that is, irregularities in band width and density (see Figure 2) are taken into account, hence, when integrating peak functions, the exact amount of protein in electrophoretic bands is quantified. The orthogonal densitometric mode determines in a single scan the protein content of a selected band in several samples run in subsequent lanes. The baseline is spectacularly flat and easy to be determined. By using known amounts of MHC it is possible to trace linear calibration curves for MHC1 and MHC2 in the range of 0.25-10 μg of each heavy chain (Figure 3). The protein amount and relative proportions of MHC1 and MHC2 in experimental samples are easily calculated by interpolation. The quantitation of MHC bands confirms the visual impression that step-one separates and concentrates more than 90% of MHC.

Figure 4 shows the analyses of myosin light chains coprecipitated with MHC and those recovered after the final step. In lanes 1-5 mixtures of EDL and soleus myosins were ran in 2.5, 5, 10, 20, 50 μg loads, respectively. Aliquots of the resuspended soleus pellets after the first precipitation step at pH 8 or 10 were loaded in lanes 8 and 10, while those of EDL were loaded in lanes 13 and 15. Note that the patterns of proteins precipitated at pH 1 (lanes 7 and 12 soleus and EDL, respectively) are very different from those of KDS-myosin precipitated at pH 8 or 10: while MHC precipitate even at alkaline pH the MLC, in particular LC1F and LC1S, remain largely in supernatants. Indeed lanes 9, 11, 14 and 16 show that light but not heavy chains precipitate after step-two from both pH 8 and pH 10 supernatants. Though densitometric scanning of this slab gave poor results due to extreme irregularity of bands, the recalculated data confirmed the visual impression (data not shown).

Discussion

Potassium and sodium dodecyl sulfate have a poor solubility at low temperature and acid pH. This fact has been used to reveal protein bands in gel slabs [14], to decrease concentration of SDS in solution [23], and recently by us to concentrate proteins from highly diluted solutions [8].

We have here extended our method demonstrating that a selective precipitation of MHC from MLC is achievable if well-guided changes in pH and temperature are intelligently employed. Indeed proteins differ in their sensitivity to this alkyl ionic detergent and this has been extensively used in the past for selective isolation of membrane proteins [16].

The interaction between protein and detergent depends by the physico-chemical properties of the SDS in water. In aqueous solutions detergent molecules are present in form of monomers and micelles. The micelles are fairly monodisperse compact aggregates, where the apolar groups of the detergent molecules are sequestered into the center and the polar groups face outwards. At low detergent concentrations only monomers are present, whereas at high concentrations both monomers and micelles exist in equilibrium. The critical micellar concentration (cmc) is a convenient but inexact parameter in the description of micellar formation, usually definable within a narrow concentration range [1, 15]. For practical purposes the cmc represent the highest monomeric detergent concentration (and thereby the highest detergent chemical potential) obtainable. The micelle size increases and the cmc decreases with increasing size of the apolar moiety of the detergent molecule and to a lesser extent, with decreasing

size and polarity of the polar groups, proteins can be incorporated into the micelles. The monomeric detergent concentration in equilibrium with these mixed micelles is lower than the cmc. When high speed centrifugation is used, the partial specific volume ($\bar{v}$) of the detergent is at least as important as the micelle size, because the buoyancy factor ($1 - \bar{v}\rho$) determines the rate of sedimentation at any density (ρ). Values of $\bar{v}$ and how they are used have been subject of studies. The $\bar{v}$ value for sodium dodecyl sulfate is 0.864 cm^3 / g. The properties of ionic detergents are strongly affected by ionic strength, nature of the counterion, temperature and pH [15]. The electrostatic interactions play a role in micelles formation: the repulsion between sulfate groups is a limiting factor, instead increasing counterion concentration result in an increase in micelle size. The magnitude of these effects depends on the type of counterion employed. Temperature also plays a role. Below the "critical micelle temperature" (cmt) monomeric detergent exists in equilibrium with solid crystalline detergent. Above this temperature, the crystals dissolve with concomitant formation of micelles. The critical micellar temperature is subjected to specific ionic effects. Potassium and other divalent cations salts of alkyl sulfates, influencing the critical micellar temperature, form insoluble crystals at room temperature. The anionic detergents are effective solubilizers only at pH above the pKa of their groups. In practice, this poses no problems for the use of alkyl sulfates, sulfonate and quaternary amines [15].

During our studies on KDS-protein precipitation we observed that some proteins differed in their pH dependent solubility, especially proteins with low pI [8]. This could be explained taking into account interactions between SDS and protein which depends also on characteristics of native protein. Its aminoacidic sequence and therefore its exposed polar and apolar groups could influence, e. g., SDS/protein ratio in denatured molecules. Furthermore it must be consider the state of SDS at different pH: at alkaline pH the heads of this ionic detergent are negatively charged increasing the cmc, that is, a lot of monomers are present, therefore lowering micellar size [15]. In this situation room temperature (20-25° C) is above the critical micellar temperature, but addition of potassium salts changes cmt so that a large proportion of KDS became monomeric and therefore insoluble above 20° C.

Potassium salts discriminate also MHC from MLC: the amount of SDS bound to MHC increases at alkaline pH causing a higher instability of the heavy chain in presence of KCl. On the other hand the SDS amount bound to light chains and other contaminating proteins does not change with pH, so that at pH 8 or 10 the stability of MLC is decreased. Indeed at alkaline pH the bulk of KDS monomers precipitate, that is, the large majority of free KDS (Sandri et al., manuscript in preparation) and more than 90% of MHC. On the other hand when precipitation is performed at pH 1 MHC, MLC and all the contaminating proteins are precipitated together with free KDS in a voluminous pellet which is difficult to handle for further analyses, e. g., the KDS imposes a relatively large volume of resuspension medium. When a two-step precipitation procedures is used, the bulk of myosin light chains, which remains in solution after the first step, is precipitated in a very small pellet which contains a small amount of free KDS.

The method here described effectively separate MHC from MLC, but purity of the two fractions strongly depends on purity of the native myosin. Indeed actin and other contaminants are present in both MHC and MLC fractions, but with a worse effect on the latter. If myosin contaminants account for 5% in weight, since LC represent 10% in weight of myosin and almost all the non-MHC proteins are in the LC fraction, the concentration of total LC increases in the sample from around 9% to 60%. We recommend this easy and inexpensive protocol as a very useful step in isolation of MLC, which must be followed by other purification procedures, e. g. HPLC [11] or Electroendosmotic Preparative Gel Electrophoresis [22]. As to the preferential separation of LC1 at pH 8, the stronger interaction of LC2 with MHC probably explains the shown results, further studies are in progress to take advantage of these preliminary observations.

We would bring the attention of the reader also to the easiness, rapidity and reliability of orthogonal densitometry, which, combined with the usual parallel scanning mode and the use of several known amounts of protein markers in the same slab, become a reliable method to absolutely quantitate SDS-denatured proteins. One of the critical advantage of orthogonal densitometry is the elimination of the well known problems resulting from background drifting in standard gel densitometry; indeed the empty interband gel offers a safe blank to which refer peak baseline.

Finally we stress that the method here described, though assembled from established procedures, for the logical extensions we introduced, became a new reliable protocol to separate and quantitate by SDS PAGE myosin light and heavy chains. It seems to be especially useful when shortness of material, as is the case of muscle biopsies or tissue cultures [4, 5, 9], make the use of standard methods of protein purification unpractical or useless. We leave to the reader's creative imagination the obvious adaptations that allow to apply the method to other proteins, e. g., to acidic protein or to those rich in apolar aminoacids.

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