Separation and Identification of Porcine Sarcoplasmic Proteins by Reversed-Phase High-Performance Liquid Chromatography and Polyacrylamide Gel Electrophoresis

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Water-soluble proteins from postrigor porcine longissimus muscle were extracted and separated by reversed-phase high-performance liquid chromatography (RP-HPLC). Fourteen protein components, including phosphorylase *b*, phosphoglucose isomerase, pyruvate kinase, phosphoglucomutase, enolase, actin, creatine kinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphoglycerate mutase, triose phosphate isomerase, myokinase, and myoglobin, were identified. Column effluent peaks were collected and identified by comparison with standards and by molecular weight in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). With optimal separation, 10 of the proteins were resolved as nearly homogeneous fractions. The remaining proteins were partially resolved in three peaks. RP-HPLC provides a rapid method for analysis of water-soluble muscle proteins.

An aqueous or low ionic strength extract of skeletal muscle contains a complex mixture of nucleotides, amino acids, peptides, and proteins, primarily glycolytic and mitochondrial enzymes, pigment, and sparingly soluble myofibrillar components such as actin. The enzymes of glycolysis comprise the bulk (more than two-thirds) of sarcoplasmic proteins (Czok and Buecher, 1960; Scopes and Stoter, 1982). These proteins have been fractionated by numerous precipitation procedures (Czok and Buecher, 1960; Scopes and Stoter, 1982; Petell et al., 1982), starch gel electrophoresis (Hartshorne and Perry, 1962; Aberle and Merkel, 1966; Scopes, 1968), and SDS polyacrylamide gel electrophoresis (Scopes and Penny, 1971). Chromatographic separations of bovine, rabbit, chicken, and porcine sarcoplasmic proteins have been reported using ion-exchange (Fujimaki and Deatherage, 1964; Rampton et al., 1965), affinity-elution (Scopes, 1977), and size-exclusion chromatography (Davis and Anderson, 1984). While these chromatographic methods are often useful preparative techniques, they lack the resolution achieved by electrophoresis.

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a rapid method for separating amino acids, peptides, and proteins based primarily on hydrophobicity (Regnier and Gooding, 1980). The amino acid composition of a polypeptide chain or exposed residues of protein determines the order of elution. A typical reversed-phase separation uses a binary solvent system consisting of an aqueous primary solvent and a hydrophilic secondary solvent or organic modifier. When the hydrophobicity of the secondary solvent is varied or the gradient of the mobile phase modified, protein mixtures have been successfully separated with alkylsilane bonded columns (Mahoney and Hermodson, 1980; Lewis et al., 1980; Pearson et al., 1982; Lewis and DeWald, 1982).

Though nominally the same, commercial columns of like description often differ in type of silica support, end-capping, and total carbon load (Pearson et al., 1982). To optimize the separation of porcine sarcoplasmic proteins by RP-HPLC, we compared two similar commercially available columns. Proteins were identified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The objective of the study was to develop a rapid chromatographic method for the separation of sarcoplasmic proteins. A useful application of the technique is measurement of proportion or solubility of individual sarcoplasmic fractions in a muscle sample.

MATERIALS AND METHODS

Muscle Sample Preparation. Longissimus muscle samples from the 10th thoracic to 3rd lumbar vertebral location were removed 24 h postmortem from one side of each of six pigs (100 kg) exhibiting normal pH fall (ultimate pH 5.5). Muscles were trimmed of visible fat, connective tissue, and adjacent tissue from other muscles, double-wrapped in Kraft poly freezer paper, and frozen and stored at −20 °C until analyzed.

Muscle slices (25 mm thick) and cores from the slices (20 mm) were removed frozen and thawed to 4 °C. Cores were weighed (approximately 10 g) and homogenized in a Sorvall omnimixer (450 mL) at high speed for 90 s (three repetitions of 30 s on 10 s off) with 2.5 times (w/v) glass-distilled water that had been filtered through a 0.5-μm filter (Aldrich Chemical Co., Milwaukee, WI). Homogenates were centrifuged at 18000*g* at 4 °C for 30 min, and the supernatant was filtered through Bio-Rad Prep disc 0.5-μm filters. The clear extract was stored at 4 °C until analyzed but not longer than 24 h.

SDS-PAGE. Electrophoresis was performed with the discontinuous buffer system described by Laemmli (1970). Slab gels (250 × 150 × 0.8 mm; 29.5:0.8 acrylamide/bisacrylamide) were cast to produce 12% resolving gels. Samples were solubilized in equilibration buffer (2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.675 M Tris-HCl, pH 6.8, and a small amount of bromophenol blue). A maximum of 21 samples were loaded per gel. Electrophoresis was performed overnight (5–7 mA/gel, room temperature) or for several hours (28–30 mA/gel, 4 °C). Gels were stained with a 50:12:0.2 methanol/acetic acid/Coomassie Brilliant Blue R solution and destained with 20:12 ethanol/acetic acid.

Chromatographic Procedure. Chromatography was performed on a Model 332 gradient liquid chromatograph including two Model 110A pumps, Model 420 microprocessor-controller, Model 210 sample injector and mixing

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chamber, and a Model 153 UV–vis detector, all from Beckman.

The primary solvent (solvent A, pH 2.25) consisted of 0.1% trifluoroacetic acid (TFA) in glass-distilled water; secondary solvent (solvent B) was acetonitrile (AcN) containing 0.07% TFA. TFA and AcN were obtained from J. T. Baker Chemical Co., Glen Ellyn, IL, and water was from Aldrich. All reagents were HPLC grade.

Columns were C-18, large-pore (300 Å), small particle size, 4.5 × 250 mm, RP-P Synchropak, Synchrom, Linden, IN, and Vydac 218 TP 54, Phenomenex, Palo Verde Estates, CA. A 12-cm precolumn (RSC bulk support, Anspec, Ann Arbor, MI) was prepared and used with both columns for all analyses. The precolumn was repacked as needed.

For most analyses undiluted aqueous extracts (volume of 20 μL) were injected by syringe into a 20-μL calibrated loop; larger sample volumes were injected into a 100 μL calibrated loop. Dilutions were made by solubilizing the extract in solvent A. Samples were injected at 0% solvent B and at a flow rate of 1.8 mL/min. A steep gradient brought the mobile phase to 35% solvent B in 5 min, and proteins were eluted from the column in a linear gradient of 35–60% solvent B in 55 min. Reported AcN concentrations represented precolumn gradient composition. Actual AcN concentrations within the column and in the detector are less, due to a delay between gradient formation and protein elution and detection. After sample elution, the mobile phase was increased to 100% solvent B and the column equilibrated for 10–15 min. The column was reequilibrated prior to the next sample injection. Absorbance was monitored at 223 nm using 0.02–2.0 AUFS. Eluent fractions corresponding to absorbance peaks were collected and lyophilized or dried under vacuum for electrophoretic analysis. Chromatograms were photocopied onto uniform paper, and the peak area at 223 nm was expressed as cutout peak weight; non-base-line separations were determined by the dropline method.

Standards. Purified enzymes including phosphorylase *b*, phosphoglucomutase, phosphoglucose isomerase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, enolase, and creatine kinase from rabbit muscle and lactate dehydrogenase and myokinase from porcine muscle were obtained from Sigma.

RESULTS AND DISCUSSION

Protein Identification in SDS-PAGE. Sarcoplasmic protein identifications for major staining bands (Figure 1) were based on published subunit molecular weights (Weber and Osborn, 1969; Scopes and Penny, 1971; Scopes, 1977) and comparison of the mobility of extracts with purified rabbit and porcine muscle protein glycolytic enzymes in SDS-PAGE. Observed migration of bands in SDS-PAGE corresponded to the expected pattern and published subunit molecular weights for these proteins, with the exception of enolase. The reported value of 41 000 for rabbit muscle enolase (Scopes and Penny, 1971) does not correspond to its migration in SDS-PAGE. Our results revealed that rabbit muscle enolase standard, porcine sarcoplasmic extract enolase, and the chromatographic fraction corresponding to enolase had apparent molecular weights of 45 000–47 000. Scopes (1977) also reported the anomalous behavior of chicken and rabbit muscle enolase in SDS-PAGE that suggested an apparent molecular weight somewhat greater than 45 000.

Chromatographic separations of porcine sarcoplasmic extracts were similar on Vydac and Synchropak columns (Figures 2 and 3). Elution of the first protein occurred at an AcN concentration of approximately 40%, and the last protein fraction was eluted at 54% AcN. All protein bands observed and identified in SDS-PAGE of the unfractionated extract were accounted for after chromatography (Figure 4).

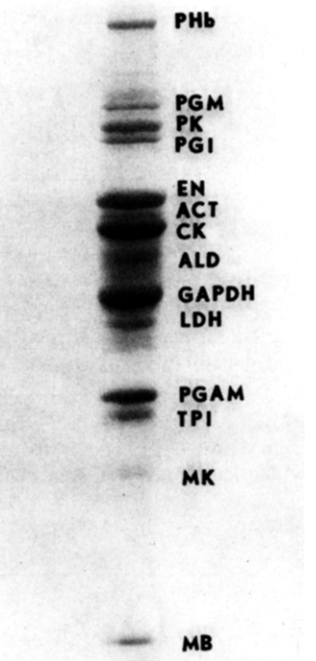

Figure 1. Electropherogram (SDS-PAGE) of separation of unheated, aqueous porcine longissimus extract with identification of bands: PHb, phosphorylase *b*; PGM, phosphoglucomutase; PK, pyruvate kinase; PGI, phosphoglucose isomerase; EN, enolase; ACT, actin; CK, creatine kinase; ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PGAM, phosphoglycerate mutase; TPI, triose phosphate isomerase; MK, myokinase; MB, myoglobin.

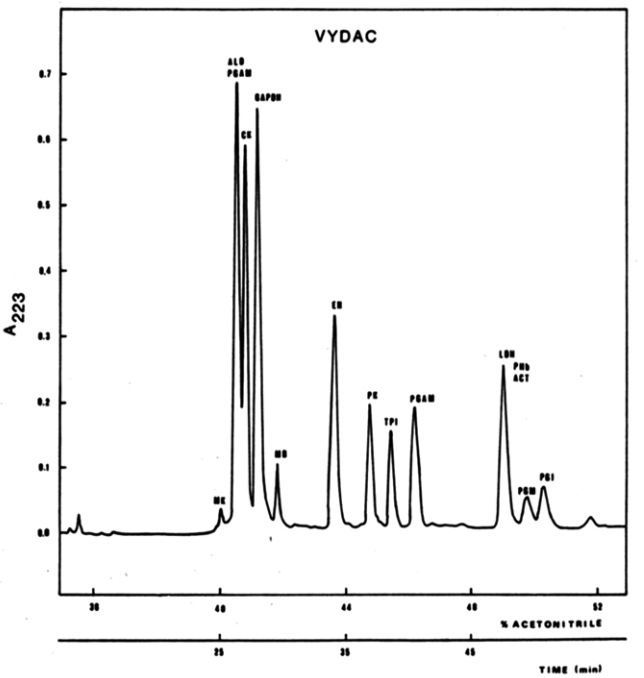

Figure 2. Chromatogram of unheated, aqueous porcine sarcoplasmic extract separation with Vydac column, sample volume 20 μL, absorbance at 223 nm, percent AcN, and time of elution. Fraction identification as per Figure 1.

Interpretation of RP-HPLC. With each column, some proteins eluted in essentially pure form, but some peaks contained two or sometimes three proteins. In one instance

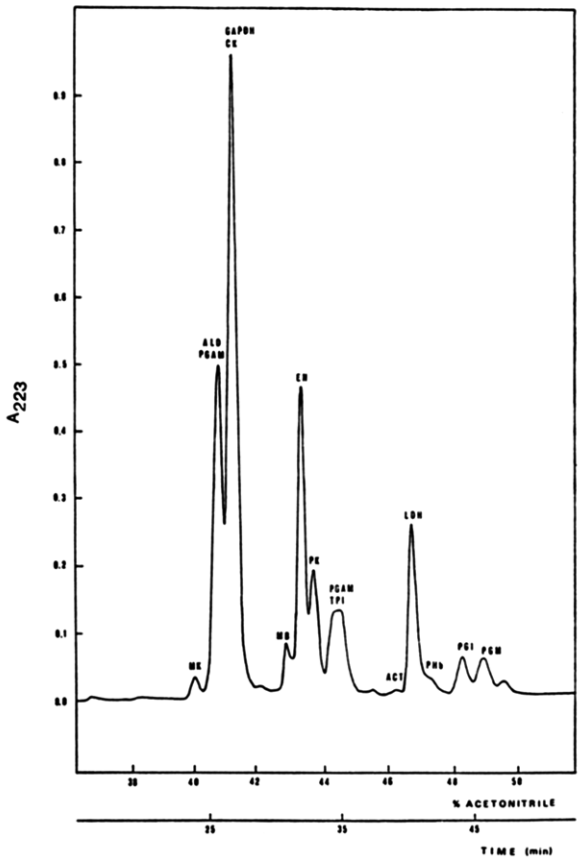

Figure 3. Chromatogram of unheated aqueous porcine sarcoplasmic extract separation with Synchropak column, sample volume 20 μL, absorbance at 223 nm, percent AcN, and time of elution. Fraction identification as per Figure 1.

components of one protein, phosphoglycerate mutase (PGAM), occurred in two peaks. Myokinase, myoglobin, enolase, pyruvate kinase, phosphoglucomutase, triose phosphate isomerase (TPI), some PGAM, and phosphoglucose isomerase eluted in nearly homogeneous form. With either column, a portion of PGAM eluted with aldolase, the greater part of PGAM eluted later as a pure fraction on the Vydac column or as a poorly resolved doublet with TPI on the Synchropak column. Creatine kinase and glyceraldehyde-3-phosphate dehydrogenase eluted together in a large predominant fraction with the Synchropak column. The same two proteins were nearly resolved on the Vydac column. Lactate dehydrogenase (LDH) could be eluted as a pure fraction from a new Synchropak column, with actin and phosphorylase *b* appearing just before and after the LDH peak, respectively. With extended column use, these three fractions tended to elute together. The corresponding fraction in the Vydac separation contained actin and phosphorylase *b* as well as LDH.

Solvent and Gradient Effects on Separation. Resolution of the complex sarcoplasmic protein mixture required a shallow linear gradient (35–60% AcN in 55 min) at a rapid flow rate (1.8 mL/min). Significantly reducing the flow rate (<1.5 mL/min) or flattening the gradient (<50 min) adversely affected resolution on both Vydac and Synchropak columns. Resolution was highly dependent on pH and TFA concentration. A shift in primary solvent pH from 2.25 to 2.5 drastically diminished resolution.

The solvent system (TFA and AcN, pH 2.25) was denaturing. An aqueous muscle extract contains proteins (enzymes), many oligomeric, in their native states. There can be little doubt that they are denatured by the harsh chromatographic solvent conditions. Nonetheless, the proteins remained soluble in both the aqueous TFA solvent and organic solvent containing TFA. The excellent solvating properties of trifluoroacetic acid apparently maintain protein and peptide solubility under conditions of pH and with organic solvents that alone would be expected to precipitate such a sample (Mahoney and Hermodson, 1980).

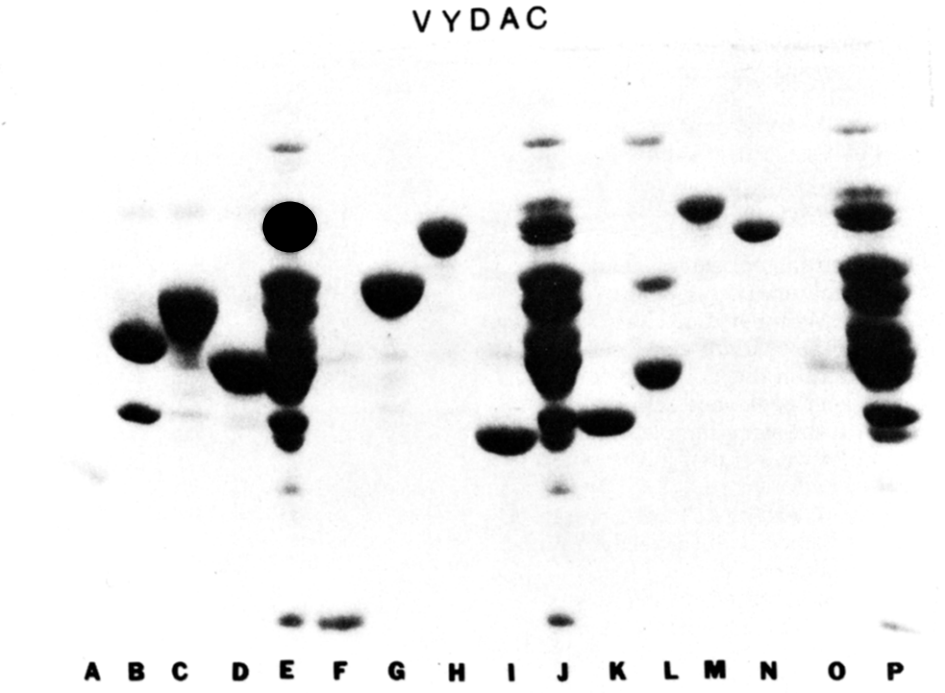

Figure 4. Electropherogram (SDS-PAGE) of HPLC fractions of aqueous porcine longissimus extract after separation on Vydac column: E, J, P, unfractioned control; B, aldolase and phosphoglycerate mutase; C, creatine kinase; D, glyceraldehyde-3-phosphate dehydrogenase; F, myoglobin; G, enolase; H, pyruvate kinase; I, triose phosphate isomerase; K, phosphoglycerate mutase; L, lactate dehydrogenase, phosphorylase *b*, actin; M, phosphoglucomutase; N, phosphoglucose isomerase; O, unidentified.

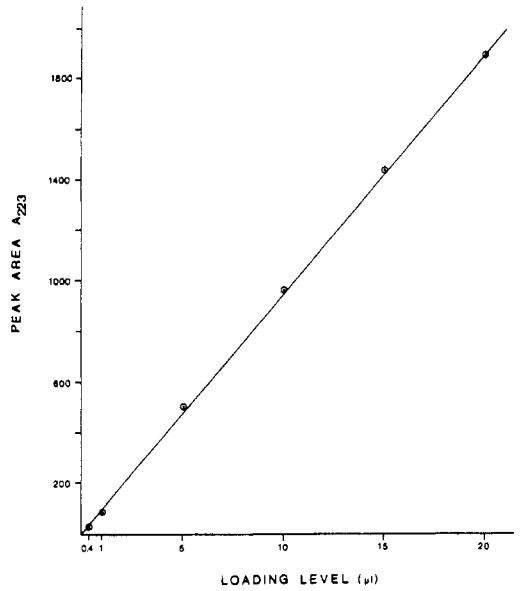

Figure 5. Relationship of peak area (absorbance at 223 nm) expressed as total cutout peak area (mg) and sample volume.

Although native oligomeric proteins are presumably denatured in the solvents for RP-HPLC, all subunits from a given protein appear to coelute, with the exception of PGAM. The monomeric proteins myoglobin, myokinase, and phosphoglucomutase eluted in separate and essentially homogeneous fractions.

In addition to the solvent system's excellent potential for resolution of complex protein mixtures, its relative transparency permits absorbance measurements to be made at or near 220 nm (Mahoney and Hermodson, 1980). This allows for greater sensitivity than absorbance measurements at 254 or 280 nm. At or near 215 nm, the peptide bond is absorbing, whereas absorbance measurements at 280 nm are a function of aromatic residues. Absorbance at the lower wavelength was more closely correlated to protein concentration (Glasel, 1978; O'Hare and Nice, 1979).

Column Properties. Chromatographic columns tend to deteriorate with use. However, the columns used in this study proved remarkably resilient. After more than 200 runs at various loading levels, the Synchropak column had some loss of resolution but was still capable of useful separations. The Vydac column, which received leses use (approximately 50 runs), exhibited little deterioration of original characteristics.

Essentially irreversible retention of proteins loaded on hydrophobic reversed-phase material occasionally occurs (Moench and Dehnen, 1978; Mahoney and Hermodson, 1980; Nice et al., 1981). With the columns and conditions used in this study, such retention did not seem to be a problem. The absence of ghost peaks and repeatability of successive separations with the same sample indicated that most or all of the samples were eluted in the course of separation.

With the Synchropak column, loading volumes differing 50-fold (0.4–20.0 μL) produced a nearly linear relationship between loading level and peak area (Figure 5).

Reversed-phase HPLC is an accepted means of separating and purifying proteins. The excellent resolution obtained with this system makes it a useful procedure for a rapid analysis of the protein components of sarcoplasmic extracts. Its possible application to other muscle proteins warrants further study.

Registry No. PHb, 9012-69-5; PGM, 9001-81-4; PK, 9001-59-6; PGI, 9001-41-6; EN, 9014-08-8; CK, 9001-15-4; ALD, 9024-52-6; GAPDH, 9001-50-7; LDH, 9001-60-9; PGAM, 9032-62-6; TPI, 9023-78-3; MK, 9013-02-9.

LITERATURE CITED

Aberle, E. D.; Merkel, R. A. "Solubility and Electrophoretic Behavior of Some Proteins of Post-mortem Aged Bovine Muscle". *J. Food Sci.* **1966**, *31*, 151–156.

Czok, R.; Buecher, T. H. *Crystallized Enzymes from the Myogen of Rabbit Skeletal Muscle*; Anfinsen, C. B., Jr., Anson, M. L., Bailey, K., Edsall, T. J., Eds; Advances in Protein Chemistry 15; Academic: New York, 1960; pp 315–415.

Davis, C. E.; Anderson, J. B. "Size Exclusion HPLC of Heated Water Soluble Bovine and Porcine Muscle Proteins". *J. Food Sci.* **1984**, *49*, 598–602.

Fujimaki, M.; Deatherage, F. E. "Chromatographic Fractionation of Sarcoplasmic Proteins of Beef Skeletal Muscle on Ion-exchange Cellulose". *J. Food Sci.* **1964**, *29*, 316–326.

Glasel, J. "Separation of Neurohypophyseal Proteins by Reversed Phase High Pressure Liquid Chromatography". *J. Chromatogr.* **1978**, *145*, 469–472.

Hartshorne, D. J.; Perry, S. V. "A Chromatographic and Electrophoretic Study of Sarcoplasm from Adult and Fetal Rabbit Muscles". *Biochem. J.* **1962**, *85*, 171–177.

Laemmli, U. K. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4". *Nature (London)* **1970**, *227*, 680–685.

Lewis, R. V.; Dewald, D. "Reverse Phase HPLC of Proteins: Effects of Various Bonded Phases". *J. Liq. Chromatogr.* **1982**, *5*, 1367–1374.

Lewis, R. V.; Fallon, A.; Stein, S.; Gibson, K. D.; Udenfriend, S. "Supports for Reverse-phase High Performance Liquid Chromatography of Large Proteins". *Anal. Biochem.* **1980**, *104*, 153–159.

Mahoney, W. C.; Hermodson, M. A. "Separation of Large Denatured Peptides by Reverse Phase High Performance Liquid Chromatography". *J. Biol. Chem.* **1980**, *255*, 11199–11203.

Moench, W.; Dehnen, W. "High Performance Liquid Chromatography of Polypeptides and Proteins on a Reversed-phase Support". *J. Chromatogr.* **1978**, *147*, 415–418.

Nice, E. C.; Capp, M. W.; Cooke, N.; O'Hare, M. J. "Comparison of Short and Ultrashort-chain Alkylsilane Bonded Silicas for the High Performance Liquid Chromatography of Proteins by Hydrophobic Interaction Methods". *J. Chromatogr.* **1981**, *218*, 569–580.

O'Hare, M. J.; Nice, E. C. "Hydrophobic High-performance Liquid Chromatography of Hormonal Polypeptides and Proteins on Alkylsilane Bonded Silica". *J. Chromatogr.* **1979**, *171*, 209–226.

Pearson, J. D.; Lin, N. T.; Regnier, F. E. "The Importance of Silica Type for Reverse Phase Protein Separations". *Anal. Biochem.* **1982**, *124*, 217–230.

Petell, J. K.; Killion, R. B.; Lebherz, H. G. *Isolation of Several Abundant Muscle Enzymes*; Wood, W. A., Ed.; Methods in Enzymology 90; Academic: New York, 1982; pp 490–497.

Rampton, J. H.; Anglemier, A. F.; Montgomery, M. W. "Fractionation of Bovine Sarcoplasmic Proteins by DEAE-cellulose Chromatography". *J. Food Sci.* **1965**, *30*, 636–640.

Regnier, F. E.; Gooding, K. M. "High Performance Liquid Chromatography of Proteins". *Anal. Biochem.* **1980**, *103*, 1–25.

Scopes, R. K. "Methods for Starch Gel Electrophoresis of Sarcoplasmic Proteins". *Biochem. J.* **1968**, *107*, 139–150.

Scopes, R. K. "Multiple Enzyme Purifications from Muscle Extracts by Using Affinity-Elution Chromatographic Procedures". *Biochem. J.* **1977**, *161*, 265–277.

Scopes, R. K.; Penny, I. F. "Subunit Sizes of Muscle Proteins as Determined by Sodium Dodecyl Sulphate Gel Electrophoresis". *Biochem. Biophys. Acta* **1971**, *236*, 409–415.

Scopes, R. K.; Stoter, A. *Purification of All Glycolytic Enzymes from One Muscle Extract*; Wood, W. A., Ed.; Methods in Enzymology 90; Academic: New York, 1982; pp 479–490.

Weber, K.; Osborn, M. "The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis". *J. Biol. Chem.* **1969**, *214*, 4406–4412.

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