ABSTRACT

This study characterized numerous cytoskeletal proteins in canine erythrocytes. Fractionation techniques were utilized to separate the blood plasma, intracellular contents, and membranes. A Bradford Assay was conducted to calculate protein concentrations for use with SDS-PAGE. Prior SDS-PAGE runs were used to critique the gels based on band characteristics. The most common problem was poor resolution. Protein identification methods used were apparent molecular weight, relative abundance, association with other fractions, and isoform analysis. Of the 10 different proteins identified, 8 were confidently identified, and three of these, spectrin, adducin, and tropomyosin, were actually doublets. The remaining two were identified with moderate confidence.

INTRODUCTION

Cytoskeletal proteins are a well-characterized subset of cellular proteins that serve a number of critical functions within cells including, but not limited to: maintaining cellular membrane stability, providing pathways for exchange of materials, assisting in cell division, and facilitating cell-to-cell recognition. This study addressed the characterization of these proteins in canine erythrocytes. Understanding the qualities of these proteins is key to understanding how cells function.

Erythrocytes were chosen because they are the simplest model available for the study of fractionation and characterization of proteins. They are the only liquid tissue which puts them already in suspension. Furthermore, they are easily lysed, which makes fractionation of the sample a relatively simple process that yields distinct fractions for study. Erythrocytes are readily available in large quantities as well. The simplicity of the erythrocyte should allow its study to shed light on similar cytoskeletal protein functions and compositions in more complex cells.

The necessity of protein in basic cell function leads to questions concerning what these proteins are and where they are located. The existence of numerous proteins requires an understanding of how differences between proteins can be measured qualitatively and quantitatively. This study specifically characterizes cytoskeletal canine erythrocyte proteins by analyzing their apparent molecular weights, relative abundances, existences in other fractions, and associations with other proteins.

MATERIALS AND METHODS

Fractionation

Three mL of whole canine blood was transferred directly to a Sorvall SM-24 centrifuge tube and placed on ice. All centrifugation was conducted using this type of centrifuge tube. The isotonic buffer was 0.9% NaCl with 5 mM sodium phosphate, pH 8, and the hypotonic buffer was 5mM sodium phosphate, pH 8. Both buffers were kept on ice. Centrifugations were conducted at low speed for 5 m at 600 x g at 4 degrees C and at high speed for 10 m at 12,000 x g at the same temperature. Suspension was facilitated through trituration, and aliquot volumes were 0.5 mL. Supernatant volumes were recorded and the supernatant discarded after each centrifugation unless noted otherwise.

The blood was suspended in 10 mL isotonic buffer and centrifuged at low speed immediately. An aliquot (#1) was taken. The pellet was resuspended in isotonic buffer and recentrifuged again at low speed. No aliquots or volumes were recorded prior to discarding the supernatant because the solution was too dilute.

The pellet was then suspended in 10 mL hypotonic buffer and centrifuged at high speed. An aliquot (#2) was taken and the supernatant volume was recorded. Resuspension and high-speed centrifugation (without obtaining aliquots) was then repeated until only the membrane "ghosts" remained. The membrane pellet was agitated and removed to an aliquot tube (#3) after discarding the supernatant.

Assay

A Bradford Assay was conducted on all three aliquots and standards. One µg/µl Bovine Serum Albumin (BSA) was mixed with the isotonic buffer to create these standards. Absorbance

was measured at 590 nm using Coomassie Brilliant Blue G-250 (as prepared by Bradford) as the reagent.

Gel Preparation

Laemmli-type gels of 7.5% T and 15% T with 2.5% C_{bis} were prepared for electrophoresis.

Sample Preparation

The sample buffer (2X concentrated) consisted of 2% SDS, 20% glycerol, 20 mM Tris-Cl (pH 8), 2 mM EDTA, 5% 2-mercaptoethanol, and a small amount of bromphenol blue as a tracking dye.

In general, protein samples were prepared to 2 mg/mL final concentration by combining them with distilled water-diluted sample buffer (1X) unless the initial protein concentrations were too low. In that case, protein samples were prepared to the highest concentration possible. Samples were denatured by immersing them for 10 m in 60 degree C water. Forty µg of samples and 2 mg/mL molecular weight standards (for Laemmli-type gel calibration; purchased from Sigma) were loaded into wells for electrophoresis.

Running of Gels

The Laemmli method (including running buffer) for SDS-PAGE was employed.

Staining and Washing of Gels

The staining dye used consisted of 0.1% Coomassie Blue dye in 1 part acetic acid:4.5 parts methanol:4.5 parts water. The gel was immersed in staining solution and agitated overnight. The washing solution was composed of 10% acetic acid and 7.5% methanol. Solutions were immersed in this solution under agitation overnight, as well.

RESULTS

Protein yield calculations revealed for future studies that the highest protein content existed in the cytosol fraction (aliquot 2; 220 mg), followed by the plasma (aliquot 1; 80 mg) and membrane (aliquot 3; 8 mg) fractions, respectively.

Gel Analysis

7.5% T (Fig. 1)

Bands in this gel in all lanes except those containing the membrane fraction of interest resolved with high clarity. The standard in lane 2, however, appeared moderately distorted in the lower quarter of the gel. Protein 'O' in lane 1 resolved partially over the area of lane 2. Lanes 4, 5, and 12 (membrane fraction) was very dark with what appeared to be a stained smear spanning the length of the separating gel from the top to the dye front. This resulted in poor protein resolution, but a few proteins were still identifiable. Each gel contained a lane with proteins from an aliquot taken immediately after the addition of hypotonic buffer to the erythrocyte pellet (lysing). The lanes containing the lysate (lane 8) and cytosol (lanes 3 and 10) fractions resolved few, if any, proteins, but a mass of what appeared to be unresolved proteins was visible at the bottom of the cytosol fractions. Mild field effects were observed in the protein bands of lane 1. This gel overall was better prepared than the 15% T gel (Fig. 2) and therefore was easier to interpret.

15% T

More pronounced field effects on both sides of this gel were observed. Asymmetrical crooked bands were found in all lanes and actually appeared amplified toward the bottom of the membrane fraction lanes where proteins stained in the shape of an arch. Resolution in lane 1 was poor with few distinguishable bands. The molecular weight standards in lanes 6, 8, and 10 did

not resolve at all, but those in lanes 2 and 5 resolved well with distinguishable bands. The membrane fraction lanes (4 and 11) appeared smeared like those in the 7.5% gel. The lysate and cytosol fractions again resolved very few proteins and had similar accumulations of unresolved proteins at the bottom of the cytosol fraction lanes.

Protein Band Analysis

Band staining intensities were characterized as low, medium, or high intensity. Bands C, 1, and 2 were used as the references for low, medium, and high intensity stains for both gels, respectively.

7.5% T

Seven membrane fraction protein bands were well-resolved in this gel. Visible protein bands on the membrane fraction were associated exclusively with that fraction. Band staining intensities were characterized as low, medium, or high intensity. Bands C, 1, and 2 were used as the references for low, medium, and high intensity stains, respectively. Membrane fraction bands were dispersed through the whole spectrum of intensities and resolvable molecular weights (Table 1). While none of the bands possessed any unusual shapes, bands A and B, as well as C and D, resembled doublets.

15% T

Four membrane protein bands were well-resolved in this gel, as well, but band H appeared to be the same as band G on the 7.5% T gel. Again, visible protein bands on the membrane fraction were associated exclusively with that fraction. Bands however, were concentrated in the middle third of the lane (Fig. 2) and were generally of low intensity with a few exceptions (Table 1). Bands J and K appeared to be a doublet, but no other major associations were noted.

Table 1. Apparent Molecular Weights and Staining Intensities for Protein Bands

Protein Band	Apparent Molecular Weight (kDa)	Staining Intensity
7.5% T		
A	>205	High
B	200	High
C	106	Low
D	102	Low
E	95	Medium
F	80	Medium
G	45	High
O	70	Very High
15% T		
H	46	Medium
I	42	Medium
J	34	Low
K	32	Low

DISCUSSION

Protein Yields

Calculated yields suggest that dramatic differences exist between the amounts and molecular weights of specific proteins in various parts of the cell. This data gives a standard of comparison by which future researchers following similar fractionation procedures can verify that the process was conducted properly. The plasma fraction contains mostly extracellular proteins including the common mammalian protein, albumin. The cytosol fraction, obtained after initial centrifugation, contains mostly free-floating intracellular proteins. Hemoglobin, by far the most abundant intracellular protein in blood cells, contains an Fe^{2+} ion in each of four heme groups thereby giving hemoglobin a higher molecular weight. These two factors combined is one explanation for the much higher yield of the second aliquot. The membrane fraction is composed of all the integral membrane proteins as well as those bound to it in some fashion. This yield is lowest because the proteins are within the surface area of the blood cell only. The erythrocyte membrane is the fraction of interest in this study.

Gel Analysis

No explanation is readily available for the complete lack of resolution of the molecular weight standards in lanes 6, 8, and 10 except that no protein was actually injected and this error went unnoticed.

Even though the 7.5 % T gel is easier to interpret than its 15% T counterpart, both have certain flaws. The field effects in both gels are caused by interference of the electric field by the spacers used to hold the glass plates in each gel cassette apart. This causes lanes at the edges to be biased to one side resulting in the poor resolution of the 15% T gel's lane 1 as well as angled bands in the outer lanes of both gels. However, the "frowning" effect associated with this

problem is not visible in higher percent acrylamide gels because the dye front washes out completely. Field effects are not completely avoidable but are lessened by ensuring that spacers fit tightly between the cassette plates.

The protein smears in the membrane fraction lanes of both gels is a product of the sample buffer component 2-mercaptoethanol, which breaks down Band 3. Band 3 is an abundant integral membrane protein responsible for anion exchange between the intra- and extracellular environments, and its breakdown causes erroneous resolution of the band, resulting in the smear and obscuring of other bands. Using 80 mM DTT (dithiothreitol) instead preserves the integrity of Band 3.

The similar molecular weights and bowl-like band shapes of Protein 'O' and Protein 4 (Bovine Serum Albumin; 66 kDa) suggest that 'O' is a canine form of albumin, a protein known to exist in high abundances in mammalian cytoplasm. Overloading well 1 is the likely cause of protein 'O''s resolution in well 2's space. Since the effect is moderate, careful concentration determinations during the assay should limit the effect.

The high hemoglobin content in erythrocyte cytosol and hemoglobin's structure are likely explanations for the poor resolution of the lysate and cytosol fractions of both gels. The hemoglobin quaternary structure denatures into two α- and two β-chain monomers. At this stage, the each monomer's molecular weight (16 kDa) is less than the minimum resolution capacity of both gels. Thus, it travels as an unidentifiable conglomeration of proteins until electrophoresis is stopped. Unfortunately, this problem is difficult to correct using 7.5% and 15% gels since denaturing the proteins is required for polypeptides to resolve properly. A possible solution is to run an even higher percentage gel to accommodate very low molecular weight proteins.

The crooked bands in the 15 % gel are the result of an uneven resolving gel surface. This suggests that the separating gel partially polymerized before the butanol overlay could smooth it out. This problem is easily corrected with a more careful preparation of the gel cassettes.

Fraction Analysis

The quality of fraction separation is vital to the success of any gel electrophoresis experimentation aimed at characterizing proteins related to a certain part of a cell. The general absence of bands of similar apparent molecular weight, relative abundance, and shape across multiple lanes suggests that each fraction contained proteins unique to that fraction. In the membrane fraction of interest, this pattern follows, indicating that membrane isolation was successful.

Protein Band Identification

7.5 % T

Bands A and B resemble the most abundant membrane protein, spectrin, a loosely wound helical protein consisting of two isoforms (α- and β-spectrin). Spectrin makes a mesh framework beneath the membrane giving the erythrocyte the structural integrity to withstand the mechanical stresses of circulation. Bands A and B are the darkest bands on the membrane fraction lane and exist as a doublet. These observations, combined with apparent molecular weights similar to those previously establish and existence only in the membrane fraction give rise to a high confidence in the identity of bands A and B as the α and β isoforms of spectrin.

Bands C and D likely represent the doublet of the actin/spectrin-crosslinking protein adducin. Its tetramer resembles four attached balloons whose strings are bound to actin and spectrin. The occurrence of spherocytosis in animals with defective adducin suggests that the protein is responsible in part for the maintenance of the biconcave disk shape. This band is not

visible on most membrane fractions because it is lightly stained and often obscured by Band 3. The breakdown of Band 3 due to the use of 2-mercapto-ethanol reveals these proteins as two very light bands representing the α and γ isoforms of adducin. This gives high confidence to the identity of bands C and D as adducin.

Band E appears to represent Band 4.1 which is responsible for linking Band 3 and glycophorin C to the actin/spectrin network. Band 4.1 is also proposed as a key protein involved in keeping the membrane intact. Past research has shown that Band 4.1 is more abundant than adducin. Band E has a more intense stain than C and D and is found only in the membrane fractions, giving high confidence that Band E represents Band 4.1.

Band F's characteristics suggest that it is the protein pallidin (a.k.a. protein 4.2). Shaped somewhat like a kidney bean, pallidin is responsible for linking Band 3 to ankyrin, a membrane protein attached to the spectrin mesh. Thus, it is one of the critical entities that attach the actin/spectrin network to the membrane. Pallidin and Band 4.1 have similar relative abundances (and therefore stain intensities) and do not exist in other fractions. Band 4.1 is known to have isoforms, and therefore, Band F might be mistaken as an isoform. This is unlikely because the molecular weights of the isoforms differ in the order of less than 10 kDa whereas the relative mobilities of Bands E and F suggest larger apparent molecular weight differences. With this other possibility in mind, the confidence in Band F as protein 4.2 is moderate.

Band G carries a high resemblance to dematin, a protein that bundles actin filaments. It is known to have two very similar isoforms, differing only by a small variance in the C-terminus of one form. On careful analysis, two distinct areas of greater stain intensity can be seen within the single stain area labeled 'G.' This facet gives a high confidence in the likelihood that Band G represents the membrane protein dematin.

15% T

Band H appears to be dematin as well, judging by its relative position compared to adjacent bands on the plasma fractions on both gels as well as by apparent molecular weight. This sets the transition point between the resolution capacities of both gels.

Band I likely represents the common helical filament protein actin that is responsible for giving the erythrocyte its durability. Its abundance is evidenced by the intensity of the stain, clearly darker than that of dematin, eliminating the possibility of Band I being a dematin isoform. The lack of other unique physical features visible in the gel yields only a moderate confidence in Band I's identity as actin.

Bands J and K, however, likely correspond to the protein tropomyosin. This protein shows up as a doublet, representing isoforms of the α-helices that bind directly to actin filaments. This possibly controls actin's ability to add or subtract subunits, thereby stabilizing the erythrocyte's membrane integrity. The very similar relative abundances of Bands J and K and actin suggest that the three are related. This observation, combined with the lack of Bands J and K in other fractions, gives high confidence to the likelihood that these bands represent tropomyosin.