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Partial Purification of Peroxiredoxin-2 From Porcine Skeletal Muscle

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Abstract: Fresh meat quality is adversely affected by protein oxidation. However, a fundamental understanding of the diverse factors that influence protein oxidation in postmortem muscle remains elusive. Peroxiredoxin-2 (Prdx2), an antioxidant protein, is more abundant in tough meat based on instrumental tenderness; however, the role of Prdx2 in postmortem skeletal muscle is unknown. Therefore, the objective was to develop a method to purify Prdx2 from the *diaphragm*, *psoas major*, and *longissimus lumborum*. Proteins soluble at low ionic strength were extracted, dialyzed, clarified, and loaded onto a Q-Sepharose anion exchange column equilibrated with TEM (pH 7.4). In all preparations, Prdx2 eluted between about 75 and 115 mM NaCl. Immunoreactive fractions were dialyzed against TEM (pH 8.0), clarified, and loaded onto a DEAE-650S anion exchange column. In all preparations, Prdx2 eluted between approximately 55 and 75 mM NaCl. Immunoreactive fractions were concentrated and loaded onto a Superose-12 size exclusion column. Prdx2 was detected between 14 and 16 mL, and these fractions were concentrated and reduced with 0.5% 2-mercaptoethanol. A final pass over the Superose-12 column was conducted, and Prdx2 was detected in 2 peaks from 11–12 mL and 15–16 mL. Fractions 15–16 were pooled and retained for further experiments. The elution profile of Prdx2 in all 3 muscles was similar. The identification of the primary protein was confirmed with liquid chromatography with tandem mass spectrometry. The purity of Prdx2 off the final Superose-12 column was approximately 33%, 52%, and 47% pure in the *diaphragm*, *psoas major*, and *longissimus lumborum*, respectively. This is the first report of a method to partially purify Prdx2 from skeletal muscle.

Key words: peroxiredoxin-2, porcine, anion exchange chromatography, size exclusion chromatography
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Introduction

Meat quality is affected by numerous biochemical events, including the rate and extent of protein oxidation. Protein oxidation in postmortem skeletal muscle can negatively affect meat tenderness development (Rowe et al., 2004a), water-holding capacity (Lund et al., 2007; Kim et al., 2010), and color (Mitacek et al., 2019) during extended postmortem aging. Fresh meat research often focuses on molecular changes caused by meat protein oxidation, including the quantification of carbonylation or other oxidative modifications (Estévez, 2011). However, there has been less emphasis on the role of endogenous proteins that may limit protein oxidation.

Peroxiredoxins are a family of thiol antioxidant proteins that exist to reduce reactive oxygen species, primarily hydrogen peroxide (H_2O_2). There are 6 isoforms of peroxiredoxins expressed in mammalian tissue that vary in localization within the cell. Peroxiredoxin-2 (Prdx2) exists in the cytosol, whereas other isoforms, such as peroxiredoxin-3, reside primarily in mitochondria (Rhee and Woo, 2011). All peroxiredoxins have a conserved reactive cysteine (C_P) found in a high-affinity peroxide binding site. In the peroxide binding site, a network of hydrogen bonds facilitates the interaction and stabilization between the peroxide substrate and renders the C_P highly sensitive to oxidation (Rhee and Kil, 2017). A subfamily of peroxiredoxins—2-Cys peroxiredoxins, which

includes Prdx2—has a resolving cysteine (C_R). After oxidation of the C_P to a sulfenic acid (C_P-SOH), an intermolecular disulfide bond forms between the C_P and the C_R on an adjacent peroxiredoxin subunit. Alternatively, the C_P-SOH can be hyperoxidized to a sulfinic acid and subsequently a sulfonic acid. The oxidation of Prdx2 impacts the form of the protein, including dimeric and decameric forms of Prdx2.

In aged pork, total Prdx2 monomer was greater in abundance in pork chops classified as tough than in chops classified as tender by instrumental star probe (Carlson et al., 2017; Schulte et al., 2020). It is proposed that increased Prdx2 abundance indicates a response to oxidative environment. Oxidation is known to limit calpain activity and postmortem protein degradation (Rowe et al. 2004b; Carlin et al., 2006, Lametsch et al., 2008). The diversity in form and abundance of Prdx2 in skeletal muscle has also been compared among pigs with different phenotypes. Two bands of Prdx2 have been observed on a non-reducing gel (Patterson et al., 2021). The molecular weight difference between the two bands was proposed to be differences in known post-translational modifications, such as glutathionylation, s-nitrosylation, acetylation, or phosphorylation (Rhee and Woo, 2020). The specific alterations of the two bands have yet to be confirmed.

Peroxiredoxin-2 reacts rapidly with hydrogen peroxide similar to other more well-characterized antioxidant proteins, such as glutathione peroxidase and catalase. However, Prdx2 is more highly expressed than glutathione peroxidase and catalase, demonstrating the significance of peroxiredoxins as antioxidant proteins (Karplus, 2015). Compared with other isoforms of peroxiredoxins, Prdx2 abundance in aged meat is associated with proteolysis and meat tenderness. Therefore, it is necessary to define the various forms and oxidation states of Prdx2 in skeletal muscle to characterize better the mechanisms of how Prdx2 impacts meat quality development and skeletal muscle growth. We hypothesize that Prdx2 abundance increases perimortem in skeletal muscle in response to oxidative stress. The connection, if any, between the oxidation state of Prdx2 and postmortem meat quality development is unknown. The application of *in vitro* studies can assist in putting the observed difference in abundance and diversity of forms of Prdx2 in context. Therefore, the objective was to develop a method for the purification of Prdx2 from porcine skeletal muscle so that a more detailed understanding of its function can be developed.

Materials and Methods

Muscle sample collection

Porcine skeletal muscle was obtained approximately 45 min postmortem from the Iowa State University Meat Laboratory. *Diaphragm* (DIA), *psoas major* (PM), and *longissimus lumborum* (LL) samples were immediately trimmed of visible connective and adipose tissue, finely minced, and immediately stored in a freezer at −80°C. Two ($n = 2$) separate purifications from all 3 muscles were conducted. The protein concentration of the elution fractions was measured with the Bradford dye-binding method (Bio-Rad, Hercules, CA).

Detection of peroxiredoxin-2 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting techniques, as described by Carlson et al. (2017) with modifications, were used to detect Prdx2 abundance in elution fractions. To detect Prdx2, 10 µL from each elution fraction were combined with 0.83 vol of Wang's tracking dye (3 mM ethylenediaminetetraacetic acid [EDTA], 3% [wt/vol] SDS, 30% [vol/vol] glycerol, 0.001% [wt/vol] pyronin Y, and 30 mM Tris-HCl [pH 8.0]) and 0.17 vol of 2-mercaptoethanol. Samples were heated for 15 min on a dry heat block at 50°C.

Peroxiredoxin-2 was detected using 15% polyacrylamide separating gels (10 cm by 10 cm; acrylamide: N, N'-methylenebisacrylamide ratio: 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.05% [vol/vol] N,N,N',N'-tetramethylethylenediamine, 0.05% [wt/vol] ammonium persulfate, and 0.5 M Tris-HCl [pH 8.8]) overlayed with a 5% stacking gel (acrylamide: N,N'-methylenebisacrylamide ratio: 100:1 [wt/wt], 0.1% [wt/vol] SDS, 0.125% [vol/vol] N,N,N',N'-tetramethylethylenediamine, 0.075% [wt/vol] ammonium persulfate, and 0.125 M Tris-HCl [pH 6.8]) with 10 lanes. Lane 1 of each gel was loaded with Precision Plus Protein All Blue molecular weight standards (Bio-Rad, Hercules, CA). The remaining lanes were filled sequentially with the prepared gel samples from each fraction and run at a constant voltage of 120 V for about 360 V/h with Hoefer 260 Mighty Small II units (Hoefer, Inc., Holliston, MA). The running buffer consisted of 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 0.1% [wt/vol] SDS.

Following electrophoresis, fractionated proteins were transferred to polyvinylidene difluoride membranes, 0.2-µm pore size (88520; Thermo Scientific, Rockford, IL) soaked in methanol for approximately 1 min before transfer. Gels were transferred onto membranes with TE-22 Mighty Small Transphor units (Hoefer, Inc.) at 90 V for 90 min at about 4°C. The transfer buffer consisted of 25 mM Tris, 192 mM glycine, 2 mM EDTA, and 15% [vol/vol] methanol. After transfer, the membranes were incubated on a rocker in PBS-Tween (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% [vol/vol] polyoxyethylene sorbitan monolaurate [Tween 20]) with 5% nonfat dry milk to block for 1 h at room temperature. After blocking, the monoclonal rabbit anti-peroxiredoxin-2 antibody (ab109367; Abcam, Cambridge, UK) was diluted 1:40,000 in PBS-Tween and incubated with the membranes overnight (approximately 15 h) at 4°C. Following primary incubation, membranes were washed with PBS-Tween 3 times for 10 min each. Goat anti-rabbit-horseradish peroxidase antibody (31460; Thermo Scientific) was diluted 1:10,000 in PBS-Tween and incubated with the membranes for 1 h at room temperature. Membranes were then washed with PBS-Tween 3 times for 10 min. Proteins were detected with a chemiluminescent detection kit (ECL Prime; GE Healthcare, Piscataway, NJ) and imaged with a ChemiImager 5500 (Alpha Innotech Corp., San Leandro, CA).

Peroxiredoxin-2 purification

The Prdx2 purification methods were partially developed based on descriptions to purify Prdx2 from erythrocytes (Lim et al., 1994; Bayer, 2015). Samples from each muscle (20 g) were homogenized separately in 3 vol (wt/vol) of ice-cold (4°C), low-ionic-strength buffer (50 mM Tris-HCl [pH 8.5] and 1 mM EDTA) using a Polytron PT 3100 (Kinematica, Lucerne, Switzerland) in two, 10-s bursts. The homogenates were clarified at 10,000 × *g* for 30 min at 4°C. The supernatant was filtered through cheesecloth and dialyzed in Spectra/Por 4 dialysis tubing with a molecular weight cutoff of 12–14 kDa (Spectrum, Rancho Dominguez, CA) against 40 vol (7 L) of 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1% 2-mercaptoethanol (TEM [pH 7.4]) overnight (approximately 15 h) at 4°C. Dialyzed extracts were clarified by centrifugation at 10,000 × *g* for 30 min at 4°C and filtered through cheesecloth. Peroxiredoxin-2 was detected in all 3 tissues using western blots (Figure 1).

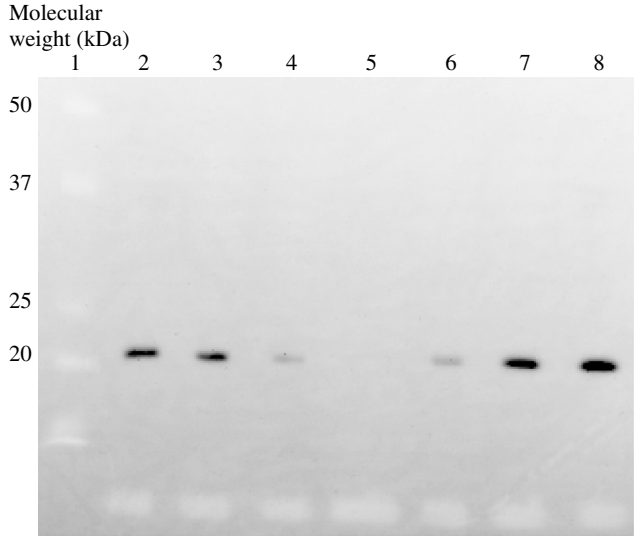

Figure 1. Representative western blot comparing the abundance of peroxiredoxin-2 (Prdx2) from dialyzed sarcoplasmic extracts of *diaphragm*, *psoas major*, and *longissimus lumborum* from one purification. Lane 1: Molecular weight standard; Lanes 2 and 8: *Diaphragm*; Lanes 3 and 7: *Psoas major*; Lanes 4 and 6: *Longissimus lumborum*; and Lane 5: Blank. Lanes 2–4 were loaded with 40 µg of total protein, and Lanes 6–8 were loaded with 60 µg of total protein.

Clarified dialysates were loaded onto separate 20 mL Q-Sepharose Fast Flow anion exchange columns (Amersham Biosciences, Piscataway, NJ) equilibrated with TEM (pH 7.4) with an ÄKTA-prime plus liquid chromatography system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Loaded columns were washed with 200 mL of TEM (pH 7.4). Proteins were eluted with a linear gradient of 75 to 500 mM NaCl in TEM (pH 7.4) over 400 mL (Figure 2A).

Immunoreactive fractions were pooled and dialyzed against 40 vol (6 L) of 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% 2-mercaptoethanol (TEM [pH 8.0]) overnight at 4°C. Dialysates were clarified by centrifugation at 10,000 × *g* for 30 min at 4°C and filtered through cheesecloth. Clarified dialysates were loaded separately on a 20 mL Toyopearl DEAE-650S anion exchange column (Supelco, Bellefonte, PA) equilibrated with TEM (pH 8.0). Each column was washed with 200 mL of TEM (pH 8.0), and proteins were eluted with a linear gradient of 0 to 500 mM NaCl in TEM (pH 8.0) over 300 mL (Figure 3A).

Immunoreactive fractions were pooled and concentrated with an Amicon Ultra centrifugal filter (Merck Millipore, Carrigtwohill, Ireland) with a molecular weight cutoff of 10-kDa at 3,000 × *g* and 4°C. Buffers for gel filtration were filtered through a bottle-top vacuum filter (0.22-µm pore size; Thermo Scientific). A Superose-12 10/300 GL size exclusion

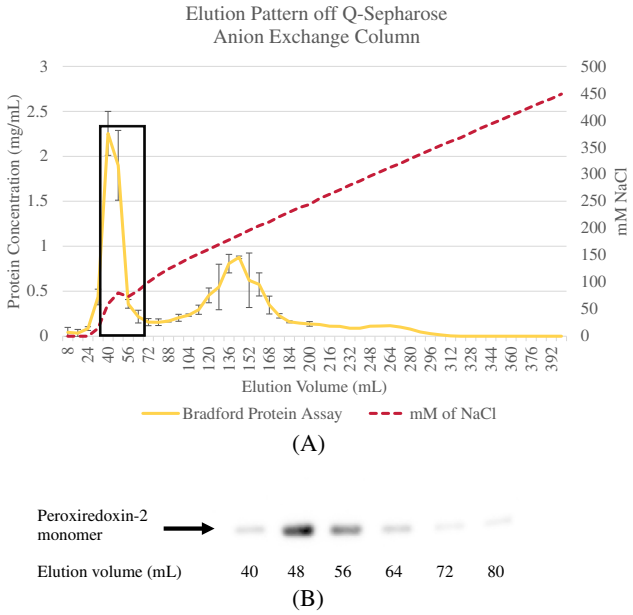

Figure 2. Representative (A) elution program and protein concentration of the elution fractions off the Q-Sepharose anion exchange column and (B) western blot of elution fractions off the Q-Sepharose column for peroxiredoxin-2 (Prdx2). The elution patterns and western blots of Prdx2 were similar, regardless of muscle; thus, only the *diaphragm* data are presented. Protein concentration is reported as an average ± standard deviation from two different preparations. The outlined region represents the immunoreactive fractions for Prdx2.

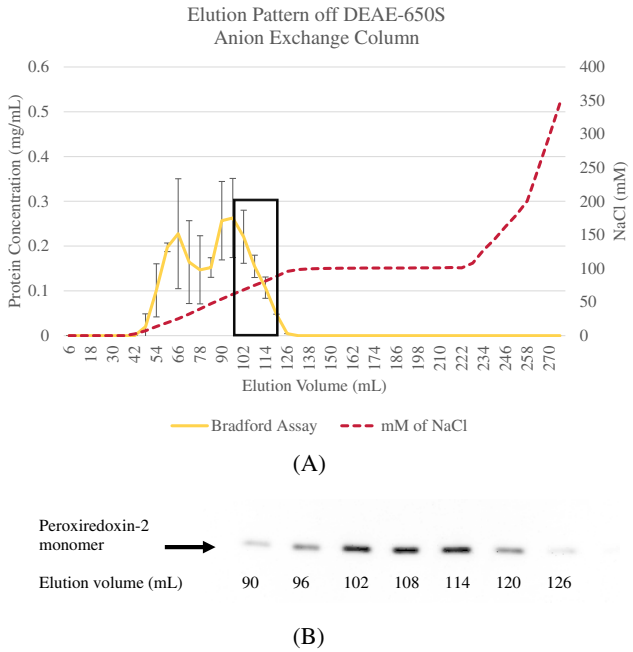

Figure 3. Representative (A) elution program and protein concentration of elution fractions off the DEAE-650S anion exchange column and (B) western blot of elution fractions off the DEAE-650S column for peroxiredoxin-2 (Prdx2). The elution patterns and western blots of Prdx2 were similar, regardless of muscle; thus, only the *diaphragm* data are presented. Protein concentration is reported as an average ± standard deviation from two different preparations. The outlined region represents the immunoreactive fractions for Prdx2.

column (GE Healthcare Bio-Sciences Corp.) was equilibrated with TEM (pH 7.4) with 150 mM NaCl. The concentrated proteins (about 1 mL, 2 mg protein) were loaded onto the Superose-12 column and fractionated with TEM (pH 7.4) with 150 mM NaCl at a rate of 0.2 mL per min in 1 mL fractions for a total of 30 mL (Figure 4A).

Elution fractions 14–16 were pooled and concentrated with an Amicon Ultra centrifugal filter (Merck Millipore) with a molecular weight cutoff of 10-kDa at 3,000 × *g* at 4°C until concentrated to a volume of less than 1 mL. 0.5% (vol/vol) 2-mercaptoethanol was added directly to the concentrated protein sample on ice for 3 h to elicit reducing conditions. The concentrated protein sample was loaded onto a Superose-12 size exclusion column equilibrated with filtered 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5% 2-mercaptoethanol with 150 mM NaCl and fractionated with the same buffer at 0.2 mL per min in 1 mL fractions for a total of 30 mL (Figure 5A).

Evaluation of purification

Protein (5 µg) from pooled fractions after each column chromatography step was used to visualize the

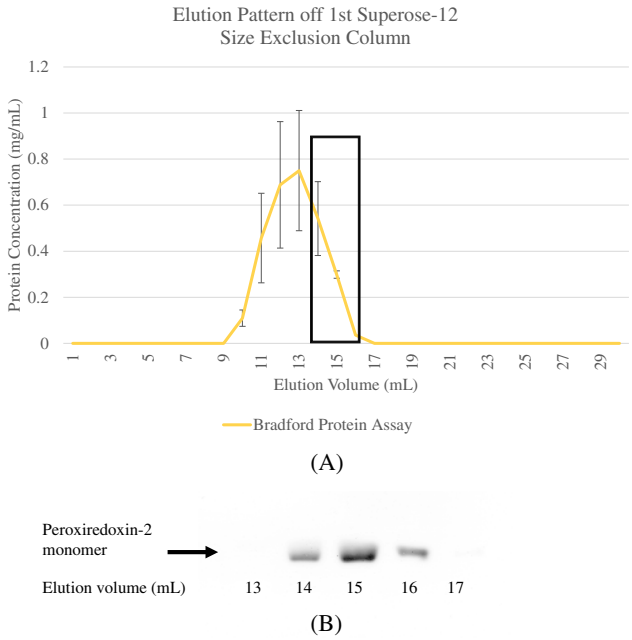

Figure 4. Representative (A) protein concentration of elution fractions off the Superose-12 size exclusion column and (B) western blot of elution fractions off the Superose-12 column for peroxiredoxin-2 (Prdx2). The elution patterns and western blots of Prdx2 were similar, regardless of muscle; thus, only the *diaphragm* data are presented. Protein concentration is reported as an average ± standard deviation from two different preparations. The outlined region represents the immunoreactive fractions for Prdx2.

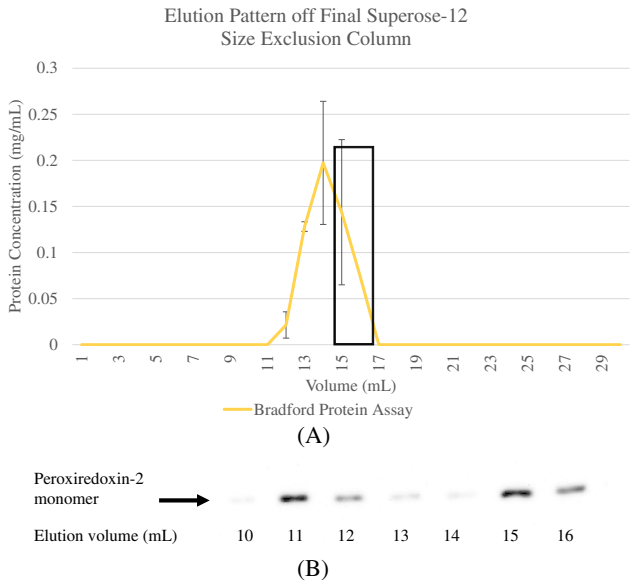

Figure 5. Representative (A) elution program and protein concentration off the Superose-12 size exclusion column and (B) western blot of elution fractions of the Superose-12 column for peroxiredoxin-2 (Prdx2). The elution patterns and western blots of Prdx2 were similar, regardless of muscle; thus, only the *diaphragm* data are presented. Protein concentration is reported as an average ± standard deviation from two different preparations. The outlined region represents the immunoreactive fractions for Prdx2.

protein profile and partial purification of Prdx2 (Figure 6). Protein (5 µg) from the final Superose-12 size exclusion column of each muscle was precipitated by the addition of 4 vol of ice-cold methanol and subsequently incubated at −80°C for 30 min. Proteins were precipitated by centrifugation at 21,694 × *g* for 30 min at 4°C. Precipitated proteins and all other samples were reconstituted with 10 µL of a solution containing 0.83 vol of Wang's tracking and 0.17 vol of 2-mercaptoethanol. Samples were heated for 15 min on a dry heat block at approximately 50°C. The prepared gel samples were loaded into 15% polyacrylamide separating gels overlayed with a 5% stacking gel with ingredients filtered through a bottle-top vacuum filter (0.22-µm pore size; Thermo Scientific), and the gels were run at a constant voltage of 120 V for 360 V/h on Hoefer 260 Mighty Small II units. Following electrophoresis, gels were stained with filtered Colloidal Coomassie Blue stain (17% [wt/vol] ammonium sulfate, 33% [vol/vol] methanol, 0.03% [vol/vol] phosphoric acid, and 0.1% [wt/vol] Coomassie G-250). Gels were destained with filtered double-distilled water, changed 2 times. Gels were imaged with a ChemiImager 5500 (Alpha Innotech Corp.) and analyzed with Alpha Ease FC software (version 3.03, Alpha Innotech Corp.). The pooled samples' purity was determined by a ratio of the 22-kDa band's densitometry compared with the sum of the total densitometry bands within a lane.

The 22-kDa band, outlined in Figure 6, was excised and submitted to the Iowa State University Protein Facility to determine the protein identity. The sample was digested with trypsin using an

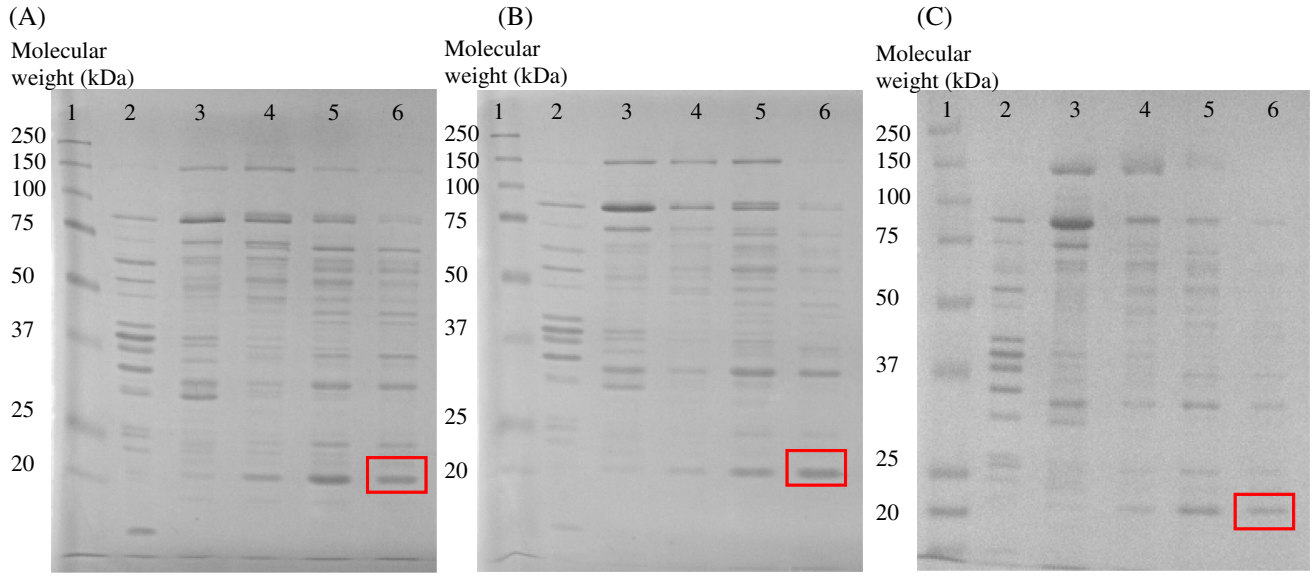

Figure 6. Colloidal Coomassie–stained 15% sodium dodecyl sulfate-polyacrylamide gels to demonstrate the protein profile and partial purification of peroxiredoxin-2 (Prdx2) from the *diaphragm* (A), *psoas major* (B), and *longissimus lumborum* (C). Lane 1: Molecular weight standard; Lane 2: Dialyzed sarcoplasmic protein extract; Lane 3: Pooled and dialyzed elution off Q-Sepharose anion exchange column; Lane 4: Pooled elution off DEAE-650S anion exchange column; Lane 5: Pooled elution off first Superose-12 size exclusion column; and Lane 6: Pooled elution off final Superose-12 size exclusion column. The outlined region represents the band excised for protein identification.

Investigator ProGest (Genomic Solutions, Inc., Chelmsford, MA) automated digestor. The peptides were separated by reverse-phase liquid chromatography using a C18 column. The peptides were eluted and introduced to the Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) and analyzed with tandem mass spectrometry. The raw spectra were analyzed with Proteome Discoverer software (version 2.4.0.305; Thermo Scientific). The fragmentation pattern of identified peptides was compared with Sequest HT and Mascot databases against *Sus scrofa* to identify proteins.

Results and Discussion

Peroxiredoxin-2 is a highly reactive thiol antioxidant protein that reacts primarily with hydrogen peroxide. In skeletal muscle, various oxidation states of Prdx2, including dimeric, hyperoxidized, and decameric, have been observed. The form and abundance of Prdx2 from skeletal muscle of pigs of differing health status and residual feed intake vary (Patterson et al., 2021). In aged pork classified as tough, total monomeric Prdx2 was greater than pork classified as tender (Carlson et al., 2017; Schulte et al., 2020). The variation in oxidation state and abundance of Prdx2 is different based on phenotype and instrumental meat tenderness classification. However, the role of Prdx2 in postmortem skeletal muscle is unknown. It is crucial to understand the role of Prdx2 to characterize the changes of Prdx2 oxidation state and abundance in order to relate better those changes to meat quality and oxidative stress responses in postmortem skeletal muscle.

Three muscles were utilized because of their muscle fiber type differences, in that the PM relies on more oxidative metabolism than the other two. In contrast, the LL depends more on glycolytic metabolism of energy (Melody et al., 2004). The DIA contains a more heterogeneous mixture of muscle fiber types but relies primarily on oxidative metabolism (Toniolo et al., 2004). As visualized in Figure 1, Prdx2 abundance was different between the sarcoplasmic extract, where the DIA and PM had a greater abundance of Prdx2 than the LL. Oxidative metabolism occurs in the mitochondria, where a significant amount of reactive oxygen species, such as hydrogen peroxide, are known to be produced (Brand, 2016). Skeletal muscles that rely on oxidative metabolism have a greater abundance of mitochondria than skeletal muscles that rely on glycolytic metabolism (Glancy and Balaban, 2011). Therefore, oxidative skeletal muscles have the potential to produce a greater amount of hydrogen peroxide. Presumably as a response to the generation of hydrogen peroxide, the more oxidative muscles—such as the DIA and PM—maintain a greater abundance of Prdx2 to mitigate oxidative damage.

Peroxiredoxin-2 has an isoelectric point of about 5.6 (Matte et al., 2013). When dialyzed against the column buffer at pH 7.4, Prdx2 would have a net negative charge and bind to an anion exchange column. In preparations from all muscles, Prdx2 eluted from the Q-Sepharose anion exchange column at approximately 75 to 115 mM NaCl, as confirmed by immunoblot analysis (Figure 2B). Proteins that did not bind to the Q-Sepharose column were removed from the protein pool through a column washing step. Figure 2A shows the protein elution pattern off the Q-Sepharose column with 2 major protein peaks. The first protein peak eluted immediately at 75 mM NaCl, and a second protein peak eluted between approximately 125 and 225 mM NaCl (Figure 2A). The protein that eluted between 125 and 225 mM NaCl contained no immunoreactive Prdx2; thus, the Q-Sepharose anion exchange column achieved a desired fractionation of proteins.

In preparations from all muscles, Prdx2 eluted at approximately 55 to 75 mM NaCl off the DEAE-650S anion exchange column, as confirmed by immunoblot analysis (Figure 3B). Bayer (2015) and Lim et al. (1994) utilized a DEAE-Sepharose anion exchange column to purify Prdx2 from erythrocytes. They report the elution of Prdx2 starting at about 160 mM NaCl and about 150–250 KCl, respectively. When using the DEAE-650S column at pH 7.4, Prdx2 eluted immediately at the start of the elution buffer up to 40 mM NaCl (data not shown). The pH was raised from 7.4 to 8.0 to achieve stronger protein binding and more efficient fractionation. By changing the pH from 7.4 to 8.0, a greater separation of proteins was achieved as no immunoreactive Prdx2 eluted between 0 and 55 mM NaCl, yet other proteins were detected in those respective fractions (Figure 3A). Therefore, the DEAE-650S anion exchange column was a partial purification step for Prdx2.

Size exclusion chromatography was employed to fractionate proteins by molecular weight. Peroxiredoxin-2 monomers have a molecular weight of approximately 22-kDa and would elute later in the elution compared with higher-molecular-weight proteins. Peroxiredoxin-2 was detected between 14 and 16 mL off the Superose-12 size exclusion column using immunoblot analysis (Figure 4B). Between 10 and

13 mL, protein was detected, yet no immunoreactive Prdx2 was observed (Figure 4A).

After the first Superose-12 size exclusion column, 0.5% (vol/vol) 2-mercaptoethanol was added directly to the pooled and concentrated Prdx2 sample. This was necessary to maintain a greater proportion of Prdx2 in a monomeric state. Peroxiredoxin-2 eluted in 2 separate peaks from fractions 11–12 and 15–16, as confirmed by immunoblot analysis (Figure 5B). It is hypothesized that the high abundance of Prdx2 and its reactive cysteines results in intermolecular disulfide bonds between Prdx2 monomers during concentration. When dimerized, Prdx2 has a higher molecular weight of approximately 44-kDa and would elute earlier than the 22-kDa monomer. Only fractions 15 and 16 were pooled and saved for further analysis.

The 22-kDa band, outlined in Figure 6, comprised approximately 33%, 52%, and 47% of the total densitometry within the pooled elution off the final Superose-12 size exclusion column for the DIA, PM, and LL, respectively. Liquid chromatography with tandem mass spectrometry analysis of the excised bands demonstrates that most of the band comprises Prdx2 (Table 1) regardless of muscle tissue. The Prdx2 preparation from the PM was specifically and uniquely assigned to Prxd2 Accession Number F1SDX9 (a 198 amino acid protein with a predicted molecular weight of 22,254 Da and an isoelectric point of 5.6).

Table 1. A summary of individual peptides of peroxiredoxin-2 identified from liquid chromatography with tandem mass spectrometry analysis.

Muscle Tissue	Protein Identification	Species	Accession Number	Coverage (%)	Identified Peptides	PSM[1]
Diaphragm	Peroxiredoxin-2	*Sus scrofa*	F1SDX9	49	EDEGIAYR EGGLGPLKIPLLADVTR GLFIIDGK KEGGLGPLK IPLLADVTR LSDYKGK LVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSK NLSLDYGVLK NLSLDYGVLKEDEGIAYR QITVNDLPVGR	938/ 2,210
Psoas Major	Peroxiredoxin-2	*Sus scrofa*	F1SDX9	82	AEEFHQLGCEVLGVSVDSQFTHLAWINTPR EDEGIAYR EGGLGPLKIPLLADVTR GLFIIDGKGVLR GVLRQITVNDLPVGR KEGGLGPLK KEGGLGPLKIPLLADVTR IPLLADVTR LVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSK LVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSKEYFSK LVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSKEYFSKHN NLSLDYGVLK NLSLDYGVLKEDEGIAYR QITVNDLPVGR QITVNDLPVGRSVDEALR YLVLFFYPLDFTFVCPTEIIAFSDR	1,354/ 2,713
Longissimus Lumborum	Peroxiredoxin-2	*Sus scrofa*	F1SDX9	51	EDEGIAYR EGGLGPLKIPLLADVTR GLFIIDGK GLFIIDGKGVLR KEGGLGPLK IPLLADVTR LSDYKGK LVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSK NLSLDYGVLK NLSLDYGVLKEDEGIAYR QITVNDLPVGR	1,145/ 2,551

[1]PSM: Peptide-spectrum matches, the total number of identified peptide sequences, including redundant peptides. Data reported as PSM of peroxiredoxin-2/total PSM identified.

Table 2. A comparison of the densitometry of the 22-kDa band to the total densitometry within each lane, the calculated fold change after each column purification step, and the total protein content from one preparation.

	Sarcoplasmic Extract			Q-Sepharose			DEAE-650S			First Superose-12			Final Superose-12		
Muscle Tissue	%[1]	TP[2]	FC[3]	%[1]	TP[2]	FC[3]	%[1]	TP[2]	FC[3]	%[1]	TP[2]	FC[3]	%[1]	TP[2]	FC[3]
Diaphragm	nd	575	-	1.3	32	-	14.4	4.3	11.1	25.1	0.5	19.3	32.6	0.17	25.1
Psoas Major	nd	750	-	2.2	35	-	15.2	3.9	6.9	18.0	0.7	8.2	51.8	0.13	23.5
Longissimus Lumborum	nd	675	-	1.6	16	-	8.6	3.2	5.4	30.2	0.4	18.9	47.2	0.11	29.5

[1]% = Percentage of the densitometry of the 22-kDa band to the total densitometry of other bands within a representative lane of muscle preparations off each column.

[2]TP = Total protein reported as milligrams of protein.

[3]FC = Fold change of the percentage purity within a lane compared with the pooled elution off the Q-Sepharose anion exchange column.

nd = not detected.

The peptides detected for the LL and DIA are consistent with the assignment to this F1SDX9 as well. However, the assignment of LL and DIA Prdx2 preparations to Prxd2 Accession Number A0A287AJ76 (a 214 amino acid protein with a predicted molecular mass of 23,360 Da and an isoelectric point of 5.1) could not be ruled out. Nevertheless, based on the behavior of all 3 preparations on anion exchange columns, gel filtration procedures, and SDS-polyacrylamide gels, it is most likely that all 3 preparations are consistent with the Prdx2 Accession Number F1SDX9.

About half of the total peptide spectrum matches (PSM) were assigned to Prdx2. The DIA band had 938 of 2,210 PSM identified as Prdx2 with a sequence coverage of 49%. The PM band had 1,354 of 2,713 PSM identified as Prdx2 with a sequence coverage of 82%. Lastly, the LL band had 1,145 of 2,551 PSM identified as Prdx2 with a sequence coverage of 51%. About half of the total PSM for the band identification were Prdx2, regardless of muscle tissue. The majority of the remaining PSM were associated with peptidase S1 domain-containing protein (Accession Number: A0A4X1V2S2).

To our knowledge, the method described here is the first to partially purify Prdx2 from skeletal muscle. The elution profiles were similar among all 3 muscles based on immunoblot and protein concentration comparisons. Purification methods were similar, yet the resulting final purity was slightly different. As mentioned earlier, Prdx2's abundance is greater in more oxidative muscles based on the immunoblot comparison from Figure 1. The final purity could be different due to muscle fiber type and the abundance of proteins specific to oxidative compared with glycolytic fiber type.

Regardless of the differences in final purity, it is clear that Prdx2's purity was greatly enhanced from the sarcoplasmic extract to the final Superose-12 size exclusion column. An approximately 25-fold increase in Prdx2 abundance relative to total protein was observed between the pooled elution from the Q-Sepharose column to the final Superose-12 column in the DIA, PM, and LL. Table 2 documents the percentage of the 22-kDa band after each column chromatography step as a ratio to total protein within the lane and the total protein content of each chromatography step. No 22-kDa band was detected in the sarcoplasmic protein extract; thus, the fold increase is more substantial than the calculation based on the product from the Q-Sepharose step. Approximately 450, 700, and 250 μg of Prdx2 were prepared from 20 g of DIA, PM, and LL, respectively. After the final Superose-12 size exclusion column, about 75 μg of Prdx2 was collected, and the percentage yield from the methods described here was about 15% to 20%, regardless of muscle tissue. Significant loss of Prdx2 occurred during the final Superose-12 size exclusion column most likely due to dimerization of Prdx2. Future efforts to improve the yield of Prdx2 are necessary; however, the products of the methods described here demonstrate the partial purification of Prdx2.

Conclusions

The methodology presented here demonstrates a reliable and repeatable approach for the partial purification of Prdx2 from porcine skeletal muscle. The final DIA, PM, and LL samples contain approximately 33%, 52%, and 50% Prdx2, respectively. Liquid chromatography with tandem mass spectrometry data validate the partial purification of Prdx2. The partial purification of Prdx2 from skeletal muscle gives a context and chance for less interference from other proteins during *in vitro* experiments. The changes in oxidation state and abundance of Prdx2 will be more easily analyzed in

a partially pure sample compared with a crude protein extract. Reliable access to a partially purified Prdx2 will make it possible to use *in vitro* methods to determine the significance of changes of Prdx2 oxidation states and abundance during the perimortem and postmortem period.

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