Meat and Muscle Biology™

Housekeeping Proteins in Meat Quality Research: Are They Reliable Markers for Internal Controls in Western Blot? A Mini Review

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Abstract: Advancements in technology and analytical methods enable researchers to explore the biochemical events that cause variation in meat quality. Among those, western blot techniques have been successfully used in identifying and quantifying the key proteins that have critical functions in the development of meat quality. Housekeeping proteins, like β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tubulins are often used as internal controls in western blots to normalize the abundance of the protein of interest. However, there are increasing concerns about using housekeeping proteins for western blot normalization, as these proteins do not demonstrate any loading differences above the relatively small total protein loading amounts of 10 μg. In addition, the interaction between these housekeeping proteins and programmed cell death processes highlights the concerns about using the housekeeping protein as the internal control in meat quality research. Moreover, recent proteomic research has indicated that the abundance of some housekeeping proteins, like β-actin, GAPDH, and tubulin, can be altered by preslaughter stress, dietary supplementation, sex, slaughter method, genotype, breed, aging period, muscle type, and muscle portion. Furthermore, these housekeeping proteins could have differential expression in meat with differing color stability, tenderness, and water holding capacity. Therefore, this review aims to examine the realities of using housekeeping proteins as the loading control in meat quality research and introduce some alternative methods that can be used for western blot normalization.

Key words: postmortem muscle, β-actin, GAPDH, tubulin, western blot
Meat and Muscle Biology 6(1): 11551, 1–15 (2021) **doi:10.22175/mmb.11551**
Submitted 10 October 2021 Accepted 23 November 2021

Introduction

Meat quality attributes such as color, tenderness, and juiciness are highly variable and are governed by the functionality of specific proteins present in meat. These quality attributes can significantly affect consumer purchasing and repurchasing decisions (Shackelford et al., 2001; Mancini and Hunt, 2005; Suman and Joseph, 2013; Neethling et al., 2017) and are economically significant. Inconsistencies in meat quality attributes have been extensively studied over the last couple of decades to understand the fundamental mechanisms behind these quality differences. Progress in technology and analytical methods have brought different mechanistic perspectives into meat quality research. These advancements have been used in research to help examine antemortem and postmortem factors, such as response to muscle fiber profile (Picard and Gagaoua, 2020; Matarneh et al., 2021), breeding and genetic selection (Warner et al., 2010; Berry et al., 2017), heat stress (Zaboli et al., 2019; Gonzalez-Rivas et al., 2020), oxidative stress (Bekhit et al., 2013), chilling methods (Zhang et al., 2019c), packaging methods (McMillin, 2017), aging processes (Kim et al., 2018b), antioxidant supplementation (Jiang and Xiong, 2016), and biomolecular interactions (Ramanathan et al., 2020), such as the interaction between lipid oxidation, myoglobin, and mitochondrial functionality.

The western blot method (Towbin et al., 1979), named after the southern blot for DNA (Burnette, 1981) and northern blot for RNA (Alwine et al., 1977), is commonly used in meat quality research for detecting and (semi-)quantifying specific proteins in given samples. Extracted proteins are separated based on molecular weight through gel electrophoresis and then transferred to a membrane. Antibodies specific to the protein of interest are used to label/identify bands containing these proteins. Key proteins that have either critical functions in the development of meat quality (Liu et al., 2019; Ma and Kim, 2020; Wang et al., 2020a) or potential biomarkers of biological pathways associated with meat quality (Chen et al., 2017; Zhang et al., 2017; Cramer et al., 2018) or posttranslational modifications (Li et al., 2016; Zhang et al., 2019a; Wang et al., 2020b) have been successfully identified and quantified using western blot.

In most cases, a comparative western blot approach relies on an equal amount of protein load in each lane. Thus, a protein quantification step is usually performed before protein loading, such as the bicinchoninic acid assay (Smith et al., 1985). As there are multiple steps between protein determination and visualization of blotted proteins, several factors can lead to unequal loading of proteins at the membrane level. Many laboratories perform immunostainings of housekeeping proteins as a standard to evaluate equal loading and compensate for potential loading differences at the level of transferred proteins.

The so-called housekeeping proteins are typically constitutive proteins required to maintain basic cellular functions and are expressed in all cells of an organism regardless of the physiological conditions. Therefore, housekeeping proteins, such as β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and tubulins are often used as internal controls in western blots to normalize the abundance of the protein of interest. The housekeeping proteins are thought to be expressed consistently (at the same levels) across experiments. However, advancements in research across different fields have raised increasing concerns about using housekeeping proteins for western blot normalization (Moritz, 2017), primarily because of the inconsistent housekeeping protein expression levels observed in samples from different treatments and tissue types. The skeletal muscle from livestock species can be heterogeneous due to antemortem and postmortem factors, and postmortem aging period can further affect proteome profile by proteolysis. Nevertheless, the use of housekeeping proteins in meat science has not yet been carefully reviewed. Therefore, this review aims to examine the challenges of using housekeeping proteins as loading controls in meat quality research and to introduce some alternative methods that can be used for western blot normalization in meat science research.

The Challenge of Using Housekeeping Protein as the Internal Control for Western Blot

β-actin, GAPDH, and α and β tubulins are the most popular housekeeping proteins used to normalize western blots. β-actin is one of the actin isoforms (42 kDa) and is a highly conserved protein involved in cell motility, structure, and integrity. GAPDH is an enzyme of about 37 kDa that catalyzes the sixth step of glycolysis, which catalyzes the conversion of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate. Both α and β tubulins are tubulin isoforms (50 kDa), which are the principal constituents of microtubules.

A clinical study by Ferguson et al. (2005) examined the variability of β-actin and GAPDH compared to the total protein levels in different human tissues. They reported up to 4-fold abundance difference in β-actin between stomach and adrenal samples and 2.5-fold abundance difference in GAPDH between lung and spinal cord samples. Later on, another research group (Dittmer and Dittmer, 2006) examined the ability of β-actin–specific antibodies to recognize differences in protein loading. They reported that at the high total protein loads (1.88 to 7.5 μg per lane) required to detect low-abundance proteins, β-actin–specific antibodies failed to distinguish differences in β-actin protein levels, indicating saturation, thus negating the ability to distinguish loading differences. The same issue was also reported in GAPDH (more than 10 μg per lane) and tubulins (more than 10 μg per lane) (Aldridge et al., 2008; Eaton et al., 2013; Thacker et al., 2016). These researchers demonstrated that β-actin, GAPDH, and α/β-tubulins are likely saturated in most experiments when used as a loading control, as they do not reflect any loading differences above the relatively small total protein loading amount of 10 μg. Taylor and Posch (2014) reviewed the western blot workflows focusing on sample preparation and data analysis for quantitative western blot. They emphasized that determining the linear dynamic range of protein loading is necessary to avoid overloading highly expressed target proteins, particularly the loading controls used to normalize the data (Taylor and Posch, 2014). Moreover,

the differential expression of these housekeeping proteins was observed in other tissues from various models (Vigelsø et al., 2014; Fortes et al., 2016; Bettencourt et al., 2020), potentially making their use unacceptable for comparisons across tissues.

In the last 2 decades, different proteomic methods, like two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Nair et al., 2016), two-dimensional differential gel electrophoresis (2D-DIGE) (Cruzen et al., 2015), label-free mass spectrometry (Yu et al., 2017), isobaric tag for relative and absolute quantitation (iTRAQ) (Xing et al., 2017), and tandem mass tag (TMT) labeling (Li et al., 2018, Zhai et al., 2020) have been applied to investigate the differentially abundant proteins and their enriched biological pathways corresponding to meat quality. Among the highlighted biological pathways, apoptotic cell death has been targeted as one of the most critical biological events in muscle-to-meat conversion and meat quality development during postmortem aging (Laville et al., 2009; Longo et al., 2015; Ma et al., 2020; Zhai et al., 2020). Ouali et al. (2006) first described the analogies between apoptosis and postmortem changes in skeletal muscle. This hypothesis was supported by the evidence that the caspase proteolytic system, the vital executioners of apoptosis, is associated with the tenderization process in pork *longissimus* and lamb *longissimus* (Kemp et al., 2006, 2009). After both morphological observation and caspase activity measurement, Becila et al. (2010) and Cao et al. (2010) confirmed the existence of apoptotic cell death in postmortem rat and beef muscle, respectively. Later on, the relationship between apoptosis and meat quality was further verified in meat from chicken (Chen et al., 2017) beef (Wang et al., 2017), and lamb (Cramer et al., 2018).

A recent proteomic study analyzed 1,297 proteins identified in 2 previous studies that evaluated protein expression across 12 different healthy human tissues (Higdon and Kolker, 2015). These authors reported that only 34 of the 1,297 proteins had a coefficient of variation less than 30% across the 12 tissues, and only 13 of those had corresponding genes that had within tissue measurement coefficient of variation less than 20% (Higdon and Kolker, 2015), which indicates that a dynamic range of protein expression exists even within same normal tissue. Meanwhile, an increasing number of researchers have pointed out that some housekeeping proteins, like β-actin, GAPDH, and tubulins, are involved with cell death processes. For example, β-actin is involved in the process of cell death, especially apoptosis, at both the protein and RNA levels (Naora and Naora, 1995; Guénal et al., 1997; Li et al., 2004; Tang et al., 2006; Wang and Li, 2007; Zhao et al., 2009; Arruda et al., 2012). In addition, many studies reported that GAPDH could affect cell death or apoptosis via a variety of biological processes (Chuang and Ishitani, 1996; Ishitani et al., 1996; Ishitani and Chuang, 1996; Saunders et al., 1997; Sawa et al., 1997; Chen et al., 1999; Cahuana et al., 2004; Hara et al., 2005; Colell et al., 2007; Tarze et al., 2007; Sen et al., 2008; Ou et al., 2010; Thangima Zannat et al., 2011). Furthermore, the involvement of α/β-tubulin in cell death was also observed in different research models (Ankarcrona et al., 1996; Martin and Baehrecke, 2004; Nolasco et al., 2005; Tracy et al., 2014; Manissorn et al., 2016; Liu et al., 2020). These discoveries bring up concerns about using housekeeping proteins as internal controls in meat quality research because of simultaneous apoptosis in postmortem muscles (Kemp et al., 2006; Kemp et al., 2009; Becila et al., 2010; Cao et al., 2010). Without ruling out the involvement of these housekeeping proteins in postmortem muscle cell death, housekeeping protein normalization could further lead to biased observations of cell death and its related biological processes responsible for meat quality. In actuality, the rapid development and application of mass-spectrometry-based technology in meat quality research has enabled researchers to find the variability of housekeeping proteins in different scenarios. The previous findings of housekeeping proteins as differential proteins and their association with meat quality will be reviewed and discussed in the following sections. The studies are also summarized in Table 1.

Housekeeping Proteins and Proteomics Research in Meat Quality Research

β-actin

Recently, Yu et al. (2018) used label-free mass spectrometry to identify muscle-specific whole proteome variations between 2 muscles with distinct muscle fiber proportion and color stability (beef *longissimus lumborum* [LL] and *psoas major* [PM] muscles) at early postmortem periods. The results showed that β-actin was more abundant in PM than LL throughout early postmortem periods (1, 12, and 24 h postmortem), and the fold change ratio (PM vs. LL) varied from 3.83 to 1.69 depending on postmortem time. In a separate study, Gulyas et al. (2018) compared the whole protein fraction

Table 1. Summary of transcriptomic and proteomic experiments reporting differential housekeeping gene expression or protein expression/modification associated with meat quality

Housekeeping gene/protein	Species	Molecular level	Target fraction	Method	Involved factor	Muscle	Postmortem collection timepoint	Reference
β-actin	Pig	mRNA	Total RNA	Real-time qPCR	Breed	LD	10 min	(McBryan et al., 2010)
β-actin	Cattle	Protein	Proteome[a]	Label-free MS	Muscle, postmortem time	LL, PM	1, 12, and 24 h	(Yu et al., 2018)
β-actin	Lamb	Protein	Proteome	2D-PAGE + MS	Breed	LD	20 min	(Gulyas et al., 2018)
β-actin	Lamb	Protein	Proteome	2D-PAGE + MS	Dietary supplement level	SM	24 h	(de Melo et al., 2020)
GAPDH	Cattle	Protein	Soluble/sarcoplasmic protein fraction[b]	2D-PAGE + MS	Sex, ultimate pH	LT	Between 24 and 48 h	(Mahmood et al., 2018)
GAPDH	Cattle	Protein	Proteome, phosphoprotein	2D-PAGE + MS	Sex, postmortem time	LD	1, 14 d	(Silva et al., 2019)
GAPDH	Cattle	Protein	Insoluble/myofibrillar protein fraction[c]	2D-PAGE + MS	Postmortem time	LT	1, 48 h	(Bjarnadóttir et al., 2010)
GAPDH	Cattle	Protein	Soluble protein fraction, Insoluble protein fraction	2D-PAGE + MS	Tenderness, postmortem time	LT	10 min, 5 d, and 21 d	(Laville et al., 2009)
GAPDH	Cattle	Protein	Proteome	2D-PAGE + MS, 2P-PAGE + MS	Postmortem time	LD	0, 12, and 26 d	(Polati et al., 2012)
GAPDH	Cattle	Protein	Sarcoplasmic/soluble protein proteins	1D-PAGE + MS,2D-PAGE + MS	Breed, postmortem time	LD	1, 7, 14, and 21 d	(Marino et al., 2014)
GAPDH	Cattle	Protein	Proteome	2D-PAGE + MS	Tenderness	LD	24 h	(D'Alessandro et al., 2012a)
GAPDH	Cattle	Protein	Phosphoprotein	2D-PAGE + MS	Tenderness	LD	24 h	(D'Alessandro et al., 2012b)
GAPDH	Cattle	Protein	Proteome	2D-PAGE + MS	Drip loss	LD	45 min	(Zuo et al., 2016)
GAPDH	Cattle	Protein	Sarcoplasmic/soluble protein fraction	2D-PAGE + MS	Color stability	LL	36 h	(Canto et al., 2015)
GAPDH	Cattle	Protein	Proteome	2D-PAGE + MS	Dietary supplement	LD	0 h	(Campos et al., 2020)
GAPDH	Chicken	Protein	Sarcoplasmic/soluble protein fraction	Label-free MS	Myopathy level	Pectoralis	0 h	(Kuttappan et al., 2017)
GAPDH	Goose	Protein	Proteome	Label-free MS	Drip loss	Pectoralis	45 min	(Zhang et al., 2019b)
GAPDH	Pig	RNA	Total RNA	Real-time qPCR	Intramuscular fat content	LD	15 min	(Hamill et al., 2012)
GAPDH	Pig	Protein	Proteome	2D-PAGE + MS	Breed	LL	24 h	(D'Alessandro et al., 2011)
GAPDH	Pig	Protein	Sarcoplasmic/soluble protein fraction	1D-PAGE + MS	Genotype	LT	20 min	(Oliván et al., 2018)
GAPDH	Pig	Protein	Proteome	Label-free MS	Muscle portion	LD	24 h	(Kim et al., 2019)
GAPDH	Pig	Protein	Sarcoplasmic/soluble protein fraction	1D-PAGE + WB	Health status	LD	0 h	(Patterson et al., 2021)
α/β-tubulin	Cattle	Protein	Sarcoplasmic/soluble protein fraction	2D-PAGE + MS	Lipid oxidation	LL	24 h	(Sayd et al., 2012)
α-tubulin	Cattle	Protein	Sarcoplasmic/soluble protein fraction	2D-DIGE + MS	Muscle, postmortem time	LL, TB	0, 7 d	(de Oliveira et al., 2019)
GAPDH, α/β-tubulin	Cattle	Protein	Myofibrillar/insoluble protein fraction	1D-PAGE + MS	Tenderness	LD	36 h	(Zapata et al., 2009)

Table 1. (*Continued*)

Housekeeping gene/protein	Species	Molecular level	Target fraction	Method	Involved factor	Muscle	Postmortem collection timepoint	Reference
GAPDH, α/β-tubulin	Pig	Protein	Sarcoplasmic/soluble protein fraction	2D-DIGE + MS	Heat stress, muscle portion	ST	0 h	(Cruzen et al., 2015)
GAPDH, α/β-tubulin	Pig	Protein	Sarcoplasmic/soluble protein fraction	2D-DIGE + MS	Heat stress, stress duration, muscle portion	ST	0 h	(Cruzen et al., 2017)
GAPDH, β-tubulin	Pig	Protein	Proteome	Label-free MS	Muscle, muscle portion	ST, SM	24 h	(Kim et al., 2018a)
β-actin, GAPDH, β-tubulin	Lamb	Protein	Proteome	2D-DIGE + MS	Slaughter method	LL	24 h	(Kiran et al., 2019)

[a]Proteome: the total amount of proteins expressed at a certain time point in a cell/tissue/animal.

[b]Soluble/sarcoplasmic protein fraction: soluble in water or low ionic strength solution.

[c]Insoluble/myofibrillar protein fraction: soluble in high ionic strength solution.

1D-PAGE = one-dimensional polyacrylamide gel electrophoresis; 2D-DIGE = two-dimensional differential gel electrophoresis; 2D-PAGE = two-dimensional polyacrylamide gel electrophoresis; 2P-PAGE = two dimensional polar polyacrylamide gel electrophoresis; LD = *longissimus dorsi*; LL = *longissimus lumborum*; LT = *longissimus thoracis*; mRNA = messenger RNA; MS = mass spectrometry; PM = *psoas major*; qPCR = quantitative PCR; SM = *semimembranosus*; ST = *semitendinosus*; TB = *triceps brachii*; WB = Warner-Bratzler.

of *longissimus dorsi* (LD) muscle proteome profile between Hungarian Merino and Tsigai sheep at 20 min postmortem, and their results indicated that Merino sheep had greater (2.6-fold) β-actin abundance than Tsigai sheep. Kiran et al. (2019) reported that β-actin quantity was approximately 6.91 times greater in nonstunned lamb LL proteome than stunned lamb. Phenotypically, the nonstunned lamb had a lower ultimate pH, water holding capacity, and Warner-Bratzler shear force than the stunned lamb (Kiran et al., 2019). A more recent study reported that the β-actin abundance in the proteome of lamb *semimembranosus* (SM) muscles at 24 h postmortem could be decreased by 10% to 15% with the supplementation of sunflower cake (de Melo et al., 2020), indicating that nutritional intervention could also impact this protein's expression. Overall, the gene expression levels and protein abundance of β-actin varied between different muscles and within the same muscle from animals with different nutritional interventions, harvest methods, breeds, and postmortem time points. These expression differences resulted from both antemortem and postmortem factors, which potentially makes using β-actin in western blot normalization an unreliable choice for standardizing postmortem muscle protein quantification.

Glyceraldehyde 3-phosphate dehydrogenase

GAPDH is a protein that has been reported to have a significant correlation with animal phenotype. For example, in a study that evaluated the proteomics of dark cutting meat, GAPDH appeared to be in greater abundance in the soluble protein fraction of *longissimus thoracis* (LT) muscle of normal steer carcasses at 24 to 48 h postmortem as compared to atypical dark cutting steer carcasses (pH < 5.9) and typical dark cutting heifer carcasses (pH > 5.9; Mahmood et al., 2018). GAPDH abundance was also positively correlated with animal age and negatively correlated with dressing percentages and ultimate pH, indicating the association between GAPDH and carcass characteristics (D'Alessandro et al., 2012a). Likewise, D'Alessandro et al. (2011) reported a greater amount of GAPDH in the proteome of pork LL muscle (more than 2-fold) from breeds with slower growth rates and higher fat deposition. At 20 min postmortem, GAPDH abundance was also shown to be differentially abundant in the soluble protein fraction of pork LT muscle between different RYR1 genotypes (NN/Nn; Oliván et al., 2018). Furthermore, Silva et al. (2019) reported that GAPDH had a greater abundance in the proteome of *longissimus* muscle from steers than bulls. In a feeding study, vitamin A supplementation elevated GAPDH abundance at 0 h postmortem in the proteome of bovine *longissimus* muscle (Campos et al., 2020). These findings suggest that GAPDH expression and abundance could be altered in muscle from animals with differing carcass characteristics, intramuscular fat content, breed, genotypes, sex, and dietary supplement.

It has been demonstrated that animal health status, harvest method, preslaughter stress, and muscle type could affect GAPDH abundance. For instance, a study

examining different lamb harvest methods indicated that GAPDH had a lower abundance (0.64-fold) in the proteome of nonstunned LL at 24 h postmortem compared to the LL muscle of stunned lambs (Kiran et al., 2019). In addition, Cruzen et al. (2015) reported that 12 h heat stress of pigs could increase the abundance of GAPDH in the sarcoplasmic fraction of white *semitendinosus* (ST) pork muscle at 0 h postmortem compared to the same muscle from pair-fed thermal neutral pigs; however, no differences in GAPDH abundance due to heat stress were observed in red ST pork muscle. Similar muscle portion-specific differential abundance of GAPDH in the sarcoplasmic protein fraction of ST pork muscle after 2 h heat stress was reported in another study (Cruzen et al., 2017). Likewise, a greater abundance of GAPDH was observed in the proteome of the light portion of pork ST and SM muscles at 24 h postmortem than the dark portion (Kim et al., 2018a). In the soluble protein fraction of pork LD muscle, GAPDH had a higher abundance in the medial region at 24 h postmortem than the anterior regions (Kim et al., 2019). Furthermore, Kuttappan et al. (2017) reported that GAPDH had a lower abundance in the soluble protein fraction of chicken breast with severe myopathic changes at 0 h postmortem than in the normal breast meat. A recent study also found an increase in oxidized GAPDH in the soluble protein fraction of health-challenged pig LL muscle at 0 h postmortem by separation on nonreducing gels. Still, no difference was found in reducing gel (Patterson et al., 2021).

Several studies have also reported the dynamic abundance of GAPDH resulting from the aging period. Silva et al. (2019) reported that GAPDH had a greater abundance in the proteome of unaged (1 d postmortem) *longissimus* muscle compared to aged (14 d postmortem) muscle. Additionally, the intact form of GAPDH decreased in abundance from 1 to 48 h postmortem in the insoluble protein fraction of bovine LT muscle (Bjarnadóttir et al., 2010). Furthermore, after both 5 and 21 days of the aging period, there was an increase in the fragments of GAPDH, a protein expected to be seen in the soluble fraction, in the insoluble protein fraction of bovine LD muscle (Laville et al., 2009), a trend that was also later observed in the proteome of same muscle during aging (Polati et al., 2012). In agreement with these previous studies, Marino et al. (2014) reported a decreased abundance of GAPDH in the soluble protein fraction of LD muscle during aging (from 1 d to 21 d) within 3 different beef cattle breeds.

Likewise, GAPDH abundance could vary in meat with differing tenderness, water holding capacity, and color stability. Zapata et al. (2009) reported that GAPDH had greater abundance in the soluble protein fraction of tender beef LD muscle at 36 h postmortem versus the tough group. Laville et al. (2009) also found that fragments of GAPDH in insoluble protein fractions had a greater abundance in tender LD than tough LD muscle at 5 d postmortem. In 2 companion studies, the first study demonstrated that GAPDH was in greater abundance (4.5-fold) in the proteome of beef LD muscle perceived as tender at 24 h postmortem than the beef LD perceived as tough (D'Alessandro et al., 2012a). In contrast, the second study reported that the phosphorylated peptide of GAPDH was found in the proteome of tough but not in the tender group (D'Alessandro et al., 2012b). Another study showed greater GAPDH abundance in the proteome of yak LT muscle with a greater amount of drip loss at 45 min postmortem (Zuo et al., 2016). GAPDH was also more abundant in the soluble protein fraction of color-stable LL steaks at 36 h postmortem than the color-labile LL steaks (Canto et al., 2015). Furthermore, goose meat with a greater amount of drip loss had a greater GAPDH abundance in the proteome at 45 min postmortem than the low drip group (Zhang et al., 2019b). Certainly, meat color stability and tenderness can be affected by muscle fiber type (Picard and Gagaoua, 2020; Matarneh et al., 2021), and the fiber types are heterogeneous in skeletal muscles. GAPDH is an enzyme associated with glycolysis, and type II muscle fiber is more glycolytic than type I muscle fiber, which could also partially account for the differing GAPDH abundance in each fractions of whole muscle proteome.

To sum up, these observations support the conclusion that GAPDH abundance and modifications can vary in postmortem muscle from the animal with the different genotypes, breed, sex, intramuscular fat content, dietary supplement, slaughter methods, heat stress, and health status. This variation can also be observed in meat with differing ultimate pH, tenderness, color stability, water holding capacity, and aging. GAPDH could undergo different levels of expression, modification, fragmentation, and denaturation by unknown mechanisms resulting from both antemortem and postmortem factors. Due to this variability, GAPDH could be an unstable internal control for western blot normalization in meat science research.

Tubulins

In meat science research, tubulins have been identified as proteins associated with different quality attributes, such as tenderness and lipid oxidation.

Zapata et al. (2009) analyzed 36 h postmortem insoluble protein fractions of beef LD muscle and linearly regressed it against Warner-Bratzler (WB) shear force (toughness) values. They observed that WB shear force was associated with the abundance of tubulins (β-2C chain, β-3 chain, α-3 chain, and α-4A chain) and their fragments. Another study reported that the abundance of a tubulin dimer (α-β) fragment in the soluble protein fraction of beef LL muscle at 30 min postmortem was positively associated with lipid oxidation at 24 h postmortem (Sayd et al., 2012).

Likewise, harvest method, heat stress, muscle type/portion, and aging period could also alter tubulin expression. In the proteome of lamb LL muscle, tubulin β-4B chains were 2.94 times more abundant in non-stunned lamb at 24 h postmortem than stunned lamb (Kiran et al., 2019). Research on pork reported that 12 h heat stress of the animal decreased the abundance of tubulins (α and β) in the soluble protein fraction of white ST muscle at 0 h postmortem compared to the thermal neutral group (Cruzen et al., 2015). However, this difference was not observed in the red ST muscle (Cruzen et al., 2015). Further studies reported that α and β tubulin abundances in the soluble protein fraction of red and white ST decreased by 2 h heat stress of the animal. Still, this difference was only observed in red ST when the heat stress was extended to 4 and 6 h (Cruzen et al., 2017). In addition, β-tubulin had differential abundance in the proteome between the light and dark portions in pork ST muscle (Kim et al., 2018a). Nevertheless, this difference did not exist in the SM muscle (Kim et al., 2018a). In the soluble protein fraction of both bovine LL and *triceps brachii* muscles, tubulin α-4A chains were less abundant at 7 d than 0 d postmortem (de Oliveira et al., 2019). In summary, as with β-actin and GAPDH, the tubulins were also differentially abundant in the postmortem muscle depending on antemortem stress and slaughter methods and in meat with differing lipid oxidation status, aging periods, muscle portion, muscle type, and tenderness. These antemortem and postmortem factors could change the fragmentation or denaturation of tubulins. Such reports of variation in abundance of housekeeping proteins are of concern and could change the context of results.

Variation in housekeeping gene expression was also reported by transcriptomic studies focusing on meat quality. A real-time quantitative PCR study (McBryan et al., 2010) tested a set of stably expressed endogenous control genes (including gene *ACTB* coding for β-actin) for messenger RNA (mRNA) expression in the porcine LD muscle from 3 pig breeds to identify potential candidate genes associated with drip loss. The results indicated that ACTB was one of the least stably expressed genes among the genes examined (McBryan et al., 2010). In addition, the transcriptomic analysis revealed that the gene coding for GAPDH was downregulated in pork LD with higher intramuscular fat content (Hamill et al., 2012).

Overall, several studies have indicated variation in abundance of housekeeping proteins, which could be a concern because it could change the interpretation and conclusion drawn from the results. Using popular housekeeping proteins like β-actin, GAPDH, and tubulins (α and β) as internal controls in meat quality research could result in poor data normalization and a confounded experimental design unable to address the researcher's questions. Researchers from different scientific fields have developed and verified alternative methods instead of using housekeeping proteins as loading controls. The following section will briefly introduce some of these available methods and their properties on polyvinylidene fluoride (PVDF) membrane.

Alternative to High-Abundance Single-Protein Controls

The purpose of any loading control is to estimate the total protein amount transferred to the blotting membrane in each lane. From this perspective, it is logical to stain the total protein on the blot to understand the total protein amount. Total protein staining represents the true loading amount more accurately than housekeeping proteins and can help account for minor technical and biological variations (Ruan and Lai, 2007; Li and Shen, 2013; Moritz, 2017). The broader dynamic range of total protein stain solves the issue of housekeeping proteins that commonly fail to show loading differences above small loading amounts (10 μg) (Moritz, 2017). Total protein staining assays based on stains like Coomassie R-350/G-250, Amido Black, Ponceau S, and Sypro Ruby or even stain-free techniques can be used as an alternative option for loading controls. In the following subsections, the properties of some of these total protein staining approaches are briefly summarized. The protocols for these not included in this section but are described in detail by Goldman et al. (2016).

Coomassie blue

Coomassie blue forms strong but noncovalent bonds by electrostatic interactions with proteins

containing the basic amino acids, arginine, and lysine (Congdon et al., 1993). This results in visible staining of protein on the membrane. Additionally, Coomassie blue does not need a fluorescence detection system to visualize the staining; its dynamic range of protein loading amount is linear and could extend from 1 to 30 μg/lane (R-350) or 5 to 50 μg/lane (G-250) (Welinder and Ekblad, 2011; Thacker et al., 2016; Moritz, 2017). The staining process is relatively quick (5 min) and does not interfere with subsequent mass spectrometry analysis (Goldman et al., 2016). However, the disadvantage of the method is its affinity with different proteins: the absorbance change per unit mass of proteins varies with the type of the protein (Congdon et al., 1993), primarily due to the differences in numbers of arginine and lysine residues among different proteins.

Ponceau S

Ponceau S is a negatively charged sodium salt of a diazo dye that reversibly binds to positively charged amino acids and nonpolar regions of proteins. Similar to Coomassie blue, fluorescence detection systems are not needed for stain visualization. Ponceau S has an almost linear dynamic range of protein loading amount, conservatively, from 5 to 50 μg/lane on PVDF membrane (Thacker et al., 2016; Moritz, 2017). Its performance is superior compared to the loading controls based on β-actin, GAPDH, α-tubulin, or total protein staining with Coomassie due to less variation across tissues and a relatively larger linear dynamic range (Romero-Calvo et al., 2010; Lanoix et al., 2012; Gilda and Gomes, 2013; Fortes et al., 2016; Thacker et al., 2016). Nevertheless, this staining method might be less sensitive to small loading differences that commonly occur in western blots because of its less steep calibration curve (Gilda and Gomes, 2013). Ponceau S staining on blotting membranes is rapid (30 s to 1 min; Goldman et al., 2016) and does not affect subsequent analytical techniques.

Amido Black

Amido Black noncovalently binds to arginine, histidine, and lysine and the terminal amino group by electrostatic interaction (Racusen, 1973). No special devices are needed for stain visualization. Amido Black has a linear dynamic range for protein loading amount from 22 to 41 μg/lane on PVDF membrane (Aldridge et al., 2008; Moritz, 2017). It was shown to be more accurate in reflecting differences in protein concentration when compared to the immunosignals of β-actin, GAPDH, and α-tubulin due to its more extensive linear dynamic range and less variability across tissues (Aldridge et al., 2008; Lanoix et al., 2012). Amido Black staining is a permanent (because of irreversible staining of proteins on membranes) and quick (1 min) stain that can be used to normalize total protein after immunodetection. Amido Black has similar sensitivity (50 ng) with Coomassie blue but higher than Ponceau S (200 ng) (Goldman et al., 2016) and does not affect subsequent protein sequencing.

Sypro Ruby

Sypro Ruby is a fluorescent stain based on an organic compound containing ruthenium that interacts noncovalently with proteins (Berggren et al., 1999). The dynamic range of protein loading amount of this total protein stain could run conservatively from 10 to 40 μg/lane on the PVDF membrane (Colella et al., 2012; Moritz, 2017). Due to the inconsistent results in its staining behavior (linear or logarithmic) from previous studies (Colella et al., 2012; Moritz et al., 2014), it is recommended to test its staining behavior with the interested dilution series and detection instrument before formal analysis (Moritz, 2017). Sypro Ruby was shown to be more accurate and stable in reflecting the differences in protein loading amount than using GAPDH and β-actin and was therefore suggested as a superior loading control compared to these housekeeping proteins (Aldridge et al., 2008; Hu et al., 2016). Unlike the protein staining methods mentioned earlier, a fluorescence imager is necessary to visualize the fluorescence emitted by Sypro Ruby staining but is relatively fast (15 min; Goldman et al., 2016). However, for unknown reasons, Sypro Ruby can inhibit the identification of cysteine and tryptophan-containing peptides for peptide mass fingerprinting using MALDI–MS, which lowers its compatibility with subsequent mass spectrometry analysis (Ball and Karuso, 2007).

Stain-free technique

The stain-free technique is based on trihalo compounds, such as trichloroethanol, incorporated into polyacrylamide gels. The staining compound can form covalent bonds with tryptophan residues of proteins under ultraviolet exposure (Ladner et al., 2004). These covalent bonds are detectable on gels and protein-transferred membranes (Colella et al., 2012; Gilda and Gomes, 2013; Gürtler et al., 2013). Conservatively, its linear dynamic range of protein loading amount comprises loading amounts between 10 and 40 μg/lane on PVDF membrane (Moritz, 2017), and many studies

have confirmed its superiority to the use of GAPDH and β-actin (Colella et al., 2012; Gilda and Gomes, 2013; Gürtler et al., 2013; Rivero-Gutiérrez et al., 2014). A fluorescent imager is needed for the detection of fluorescent emissions on the membrane. However, it has been reported that the stain-free method may not work when less than 3 to 5 μg of total protein is loaded per lane (Taylor et al., 2013). Another disadvantage could result from its staining mechanism of action. The proteins lacking tryptophan could be invisible and may lead to a bias due to its specific binding with tryptophan residues (Moritz, 2017).

Other techniques

Previous literature has also proposed other reversible total staining methods used as the internal control in western blot. Fluorescent staining methods, like epicocconone (Moritz et al., 2014), and nonfluorescent staining methods, like MemCode (Antharavally et al., 2004), have been documented. Epicocconone noncovalently interacts with sodium dodecyl sulfate (SDS) and proteins (Bell and Karuso, 2003; Mackintosh et al., 2003) with a logarithmic dynamic range of protein loading from 0.1 to 30 μg per lane on the PVDF membrane (Moritz et al., 2014). MemCode can also noncovalently react with protein, but its dynamic range and regression model of staining behavior has not been tested (Antharavally et al., 2004).

Overall, the total protein staining method should be selected depending on the researcher's question and experimental design. Researchers should avoid using staining dye with a binding mechanism that could lead to staining bias. Postmortem muscle is proteolytic, and the extent of proteolysis can be affected by multiple factors such as muscle type and aging period. Due to protein degradation during aging, there is a potential for the protein profile to shift from a higher molecular weight range to lower region. This could in turn affect the total protein signal within a region. Therefore, depending on the extraction method, researchers should account for the dynamic shift of protein profile and choose a stable range of molecular weight of total protein for signal normalization. Furthermore, it is important for researchers to be conscious of the day-to-day bias of these total staining methods. For example, it is common for a total protein staining method that the staining background has variations between different blots. This background variation could generate technical errors and further affect normalization accuracy. Nevertheless, this problem could be minimized by the incorporation of an internal reference sample onto every blot. After normalization of the interested protein to total protein staining, the obtained band intensity can be compared to this reference sample.

Conclusions

Multiple research studies have reported concerns regarding the use of housekeeping proteins such β-actin, GAPDH, and tubulins as internal control for western blots. Direct measurement of the actual amount of protein loaded by total protein staining methods can reduce technical errors and commonly used housekeeping proteins' biological variation. The broad dynamic range of total protein staining methods can also solve the limitations with a small loading amount resulting from housekeeping protein saturation. Therefore, using total protein staining in meat science western blot experiments could avoid biased results due to housekeeping protein differential abundances caused by the antemortem and postmortem factors and, therefore, can help explore the molecular mechanism behind meat quality variation.

Acknowledgements

The authors declare no competing financial interest. This manuscript was partially supported by USDA multistate grant W4177.

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