Phosphoproteomic profiling of myofibrillar and sarcoplasmic proteins of muscle in response to salting

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Abstract A phosphoproteomic profile of myofibrillar and sarcoplasmic proteins of muscle in response to salting was investigated. Myofibrillar and sarcoplasmic proteins extracted from salted meat with 0, 1, 2, 3, 4, and 5% salt for 0, 2, 4, 6, 8, and 16 h were analyzed by SDS-PAGE electrophoresis and fluorescence staining. The global phosphorylation of myofibrillar proteins in salted meat was lower than that in control muscle at 16 h of salting ($p < 0.05$), and the global phosphorylation of myofibrillar proteins in 3% salt-treated group at 16 h was the lowest. However, salting showed no significant effect on phosphorylation of sarcoplasmic proteins. Four categories of phosphorylated protein were identified by LC-MS/MS, involved in stress response (heat shock protein), glycometabolism (glycogen phosphorylase, glyceraldehyde-3-phosphate dehydrogenase), oxidation or reduction (superoxide dismutase), and others (myoglobin), the phosphorylation of which was affected by salting. Thus, salting may influence meat quality through protein phosphorylation, which regulates protein degradation and glycolysis.

Keywords: protein phosphorylation, salting, protein degradation, glycolysis

Introduction

Salting is one of the most ancient preservation methods for food (1,2). It is widely used to preserve meat and to improve the quality of meat products, such as water-holding capacity, texture, and tenderness (3,4). Salting changes the microstructure of muscle. For example, the muscle fiber diameter shrinks and the intercellular space enlarges after salting (5). Although phosphate, collagen, sodium alginate, and other ingredients are applied to improve meat quality, salt still plays a crucial role (water retention, protein solubilization, and flavor) in cured or cooked meat products (6,7).

Protein phosphorylation is one of the most ubiquitous post-translational modifications. Reversible protein phosphorylation plays an important role in protein structure, function, signaling, and regulation (8,9). The majority of proteins are phosphorylated *in vivo* in mammalian cells (10). Recent studies reveal that protein phosphorylation may affect meat quality in three ways: protein degradation, glycolysis, and muscle contraction. Some studies report that protein phosphorylation affects the degree of protein degradation, which influences meat tenderness (11,12). Glycolysis is the principal way for energy supply in postmortem muscle. Glycolysis is related to meat color, pH, and water-holding capacity. Glycolytic enzymes are

phosphorylated by protein kinases. The phosphorylation of glycolytic enzymes affects their activity and stability (13,14). In addition, protein phosphorylation affects muscle contraction (15,16), especially the phosphorylation of myosin regulatory light chain 2 (MYLC2) was found to change protein structure (16).

In recent years, the studies of phosphoproteomic methods have made it easier to research on protein phosphorylation and promoted research in protein phosphorylation. Protein phosphorylation in relation to muscle pH decline and meat tenderness was previously investigated in postmortem muscle (9,13,14). Electrical stimulation alters protein phosphorylation (17). All these studies reveal that protein phosphorylation plays a significant role in the development of meat quality. The effect of salt *in vivo* on protein phosphorylation in plants was studied and found that many proteins are salt-responsive phosphoproteins (18). Salt diet influences the phosphorylation of SPS1-related proline/alanine-rich kinase (SPAK), Na⁺/K⁺/2Cl⁻ co-transporter 1 (NKCC1) and oxidative stress-responsive kinase 1 (OSR1) *in vivo* (19,20). Salting is one of the most essential processes for meat products; however, the effect of salting on muscle protein phosphorylation is unclear. The aim of the study is to investigate the phosphorylation of myofibrillar and sarcoplasmic proteins in response to salt content and salting time.

Materials and Methods

Animals and sample preparations Eight male (not castrated), 8-month-old sheep (small-tailed Han sheep $\times$ large-tailed Han sheep) with an average carcass weight of 25–30 kg were slaughtered in a local commercial abattoir as per industrial practice. The topside muscles from both hind legs were removed immediately after slaughter and wrapped in a polyethylene film (oxygen permeation rate: $10,600 \pm 20\%$ [$\text{cm}^3/(\text{m}^2 \cdot 24 \text{ h} \cdot \text{atm})$], Shanghai NuoBang Plastics Company, Shanghai, China). They were maintained at 4°C for 24 h and then preserved at 80°C for subsequent experiments.

Before use, frozen muscles were thawed at room temperature for 3 h. Muscles from eight sheep were minced through 8-mm-pore plate and mixed together. Then the mixed muscles were divided into six groups for salting treatments with 0% (control group), 1, 2, 3, 4, and 5% green iodized salts ($\text{NaCl} > 99.1\%$, China National Salt Industry Corporation, Beijing, China) for 16 h at 4°C . During salting, samples were collected at 0, 2, 4, 6, 8, and 16 h for all treatments. The collected samples were snap-frozen by liquid nitrogen and then stored at 80°C for further research. All procedures were repeated three times.

Protein extraction Proteins were extracted as previously described (14,17,21) with some modifications. Briefly, 1 g frozen muscle was homogenized in 6 mL ice-cold homogenizing buffer (pH 8.3) including 100 mM Tris, 10 mM DL-dithiothreitol (DTT), phosphatase inhibitor PhosSTOP (a slice per 10 mL solution, Roche, Basel, Switzerland), and complete protease inhibitor (a slice per 50 mL solution, Roche) on ice using an IKA homogenizer (IKA Labortechnik, Staufen im Breisgau, Germany). The homogenate was centrifuged for 20 min at 4°C and $25,000 \times g$. The supernatant (sarcoplasmic proteins) was carefully collected without the fat layer, aliquoted (1 mL of a tube), and conserved at 80°C for analysis. The pellet (myofibrillar proteins) was dissolved in 15 mL 5% SDS solution (60°C), homogenized at 9,500 rpm for 30 s. The homogenate was incubated in a water bath of 80°C for 30 min and then aliquoted and conserved at 80°C . The concentration of myofibrillar and sarcoplasmic proteins was measured by BCA assay (Pierce Chemical Company, Dallas, TX, USA).

Gel electrophoresis and image analysis The myofibrillar and sarcoplasmic proteins were diluted to $2 \mu\text{g}/\mu\text{L}$ with ultrapure water; 100 μL of protein samples were mixed with 100 μL 2 $\times$ loading buffer (40 g/L SDS, 100 mM Tris-HCl, pH 6.8, 250 g/L glycerin, 1 g/L bromophenol blue). The samples were boiled at 100°C in the water bath for 4 min. Polyacrylamide gels were composed of 12% separating gel and 4% stacking gel, and a 5 g (5 μL) sample was loaded onto each gel lane. The conditions of the electrophoresis were as follows. The voltage in the stacking gels was 70 V, while that in the separating gels was 110 V. The electrophoresis was terminated once the dye

reached the bottom of the gel (Bio-Rad Laboratories, Hercules, CA, USA).

The gels were first stained by Pro-Q Diamond for phosphoproteins and then stained by SYPRO Ruby dye for total muscle proteins (13,14,17). Fluorescently staining gels were visualized using ChemiDocTM MP Imaging System (Bio-Rad Laboratories). Pro-Q Diamond staining gels were scanned with Pro-Q Diamond model, while SYPRO Ruby staining gels were scanned with SYPRO Ruby model. Finally, the gels that were scanned two times were stained by Coomassie Brilliant Blue R-250 to visualize the location of proteins. Quantity One 4.62 software (Bio-Rad Laboratories) was used to analyze the intensity of the protein band of the images. The relative protein phosphorylation levels were evaluated by the ratio of the intensity of phosphoproteins band (P) in the Pro-Q Diamond images to the intensity of total proteins band (T) in SYPRO Ruby images (P/T ratio) (13,17,22).

In-gel trypsin digestion and protein identification by LC-MS/MS

Proteins were identified according to Chen *et al.* (9). First, the excised protein bands were subjected to in-gel trypsin digestion. The excised gels were first washed twice for 10 min each in 1 mL ultrapure water and then washed twice for 10 min each in 1 mL in-gel digestion bleaching solution (25 mM NH_4CO_3 , 50% acetonitrile). Gels were shrunk in 1 mL 100% acetonitrile. The proteins were incubated in the water bath by 10 mM DTT at 56°C for 1 h and then incubated by 55 mM iodoacetamide at room temperature for 45 min in the dark after removing unabsorbed DTT. Gel pieces were subsequently washed twice for 10 min each by 25 mM NH_4CO_3 , washed twice for 10 min each by a bleaching solution, and dehydrated by 100% acetonitrile. The dried gel pieces were incubated in the trypsin solution (1 $\mu\text{g}/\mu\text{L}$ of trypsin dissolved in 25 mM NH_4CO_3 , Roche) on ice for 1 h before adding 25 mM NH_4CO_3 . The proteins were digested at 37°C overnight in the water bath. The digestion was stopped by 0.1% trifluoroacetic acid.

The identification was performed by an LC-MS/MS instrument (Thermo Q-Exactive; Thermo Fisher Scientific, Waltham, MA, USA). The raw spectral data obtained from the mass analyzer were extracted as peak lists by Thermo Proteome Discoverer 1.3.0.339 (Thermo Fisher Scientific). The peak lists were matched against the UniProt database by MASCOT (2.3.01). The search criterion parameters are as follows: enzyme, trypsin; one maximum missed cleavage; mass values, monoisotopic; fixed modification, carbamidomethyl (C); variable modification, oxidation, Gln $\rightarrow$ Pyro-Glu (N-term Q); and peptide mass tolerance, 15 ppm. For the assignments of all proteins, two distinct peptides should be identified for one protein at least.

Statistical analysis In our study, all statistical analyses were carried out by IBM SPSS Statistic 21.0 (SPSS Inc., Chicago, IL, USA). The difference was analyzed by the analysis of variance (ANOVA) test. Reported *p* values were evaluated at a 5% significance level.

Results and Discussion

Global phosphorylation of myofibrillar proteins with salting time

The amount of salt added in traditional meat processing is typically 2% (23). Therefore, we chose 2% salt as the experimental dose to profile protein phosphorylation during meat salting. As shown in Fig. 1A, a total of 23 bands of myofibrillar proteins were detected by Quantity One and selected for analysis. The global phosphorylation level of proteins was as shown in Fig. 1C. The P/T ratio of each sample was evaluated by the ratio of the intensity of all bands in Pro-Q Diamond images to the intensity of all bands in SYPRO Ruby images. The P/T ratio of control muscle increased from 0 h to 2 h and then decreased slightly with no significant difference during the whole salting process ($p>0.05$). Conversely, the P/T ratio of the salted meat with 2% salt significantly decreased from 0 h to 16 h ($p<0.05$).

The result showed that global phosphorylation level of myofibrillar proteins significantly decreased ($p<0.05$) during the salting development after adding 2% salt, whereas it did not change significantly in the control group. The global phosphorylation levels of the salted group were lower than those of the control group, which indicated that salt

affects protein phosphorylation. The global phosphorylation level at 16 h was the lowest point during the salting development; thus, the influence of salt on the phosphorylation of muscle proteins was further studied by adding different concentrations of salt.

Phosphorylation of myofibrillar proteins in response to salt

The phosphorylation results of myofibrillar proteins with different salt concentrations are shown in Fig. 2. Salt concentrations of 2% and higher significantly reduced myofibrillar protein phosphorylation after meat was salted for 16 h when compared to the control group, with meat salted by 3% salt having the lowest protein phosphorylation (Fig. 2C), showing that salt influences protein phosphorylation during meat processing.

The P/T ratios of individual protein bands are shown in Fig. 2D. The P/T ratio of bands 8, 16, 19, and 23 was greater than 1; hence, these proteins were highly phosphorylated. In general, the protein phosphorylation level of most bands from salted meat was lower than that of the control group, except of bands 16 and 20, which showed an increase in protein phosphorylation after salting.

Fig. 1. Phosphorylation of myofibrillar proteins during salting. (A-B) SDS-PAGE analysis of phosphorylated proteins by Pro-Q Diamond staining (A) and total myofibrillar proteins by SYPRO Ruby staining (B). (C) Quantification of global protein phosphorylation levels of myofibrillar proteins at different time points during salting. Different letters indicate significant difference with salting time ($p<0.05$). Asterisk (*) shows significant differences between the 0% salt and 2% salt groups in the same salting time ($p<0.05$).

Fig. 2. Phosphorylation of myofibrillar proteins in response to salt concentrations. (A-B) SDS-PAGE analysis of phosphorylated proteins by Pro-Q Diamond staining (A) and total myofibrillar proteins by SYPRO Ruby staining (B). (C) Quantification of global protein phosphorylation levels of myofibrillar proteins from meat salted at different salt concentrations. (D) Protein phosphorylation levels of nine individual protein bands. Different letters indicate significant differences ($p < 0.05$).

Fig. 3. Phosphorylation of sarcoplasmic proteins during salting. (A-B) SDS-PAGE analysis of phosphorylated proteins by Pro-Q Diamond staining (A) and total sarcoplasmic proteins by SYPRO Ruby staining (B). (C) Quantification of global protein phosphorylation levels of sarcoplasmic proteins at different time points during salting.

Salting decreases phosphorylation of myofibrillar proteins

Myofibrillar proteins are structural proteins responsible for sarcomere structure integrity and muscle contraction, both of which are related to meat tenderness. Salting improved meat tenderness (24). Many researchers found that protein phosphorylation may affect meat quality (9,13,17). Our study showed that the global phosphorylation level of myofibrillar proteins in salted meat was reduced. This is consistent with the literature, which reports that protein phosphorylation affects meat tenderness and that tender meat has lower protein phosphorylation (9,25). The phosphorylation of myofibrillar proteins reduced the degradation of these proteins by calpains, such as actin binding protein, troponin T (TnT), and troponin I (11,12,26). In our study, the phosphorylation of myosin heavy chain (band 3), myosin binding protein C (band 4), and actin (band 11) was reduced by salting; these bands are related to tenderness. According to all the studies, we speculated that the relatively low phosphorylation level of myofibrillar proteins in salted meat made them more easily degraded than the control group and then improved meat tenderness.

Rigor or muscle contraction shortens sarcomere length and decreases meat tenderness. Many phosphorylated proteins identified in muscle are related to sarcomere function that affects meat quality; thus, they speculated that protein phosphorylation may be related to muscle contraction and quality changes (14). The phosphorylation of myosin light chain (MLC) enhances the intensity of contraction (16). Our result showed that the phosphorylation of MLC (Fig. 2D, band 23) was reduced by salting, which may inhibit muscle contraction and further affect meat tenderness, providing another mechanism that salting increases meat tenderness.

Global phosphorylation of sarcoplasmic proteins with salting time

A total of 21 sarcoplasmic protein bands were detected to be phosphorylated in meat (Fig. 3A). Different from myofibrillar proteins, salting showed no significant effect on the phosphorylation of sarcoplasmic proteins. The P/T ratios of both the control muscle and salted meat with 2% salt showed no significant differences ($p > 0.05$) at different salting time points, though an increasing trend in P/T ratio with salting time might exist in the salted meat (Fig. 3C).

Fig. 4. Phosphorylation of sarcoplasmic proteins in response to salt concentrations. (A-B) SDS-PAGE analysis of phosphorylated proteins by Pro-Q Diamond staining (A) and total sarcoplasmic proteins by SYPRO Ruby staining (B). (C) Quantification of global protein phosphorylation levels of sarcoplasmic proteins from meat salted at different salt concentrations. (D) Protein phosphorylation levels of 15 individual protein bands.

Table 1. Identification of phosphorylated sarcoplasmic proteins by LC-MS/MS

Protein names ¹⁾	Band No. ²⁾	Accession No. ¹⁾	Species ³⁾	MW (Da) ⁴⁾	Score ⁵⁾	MP ⁶⁾	Sequence Coverage ⁷⁾	Function
Acetyl-CoA carboxylase 1	1	Q28559	OS	266736	67	3	2%	Biotin enzyme
Uncharacterized protein	3	F1MHT1	BOS	176199	19863	769	69%	
Glycogen phosphorylase, muscle form	5	O18751	OS	97702	44666	1713	77%	Glycosemetabolism
Hydroxyacyl-coenzyme A Dehydrogenase/ 3-ketoacyl-coenzyme A Thiolase/ enoyl-coenzyme A hydratase (Trifunctional protein) alpha subunit	6	B6UV59	OS	83926	2687	79	50%	Coenzyme binding
Heat shock protein alpha	6	A8DR93	OS	85077	1181	37	34%	Stress response
Heat shock protein 70	7	H2DGR2	OS	70536	5377	207	58%	Stress response
Heat shock protein 70	7	H2DGR0	OS	70471	5208	199	49%	Stress response
HSP70	7	H9LHX6	OS	69673	1130	38	8%	Stress response
Malic enzyme	8	B5SZL5	OS	64601	705	23	61%	Glycose metabolism
AMP-activated protein kinase alpha2 subunit	8	A8D245	OS	62833	50	1	9%	Glycolysis
Protein disulfide isomerase family A member 3	10	C6JUQ0	OS	57379	546	24	39%	Rearrangement of -S-S- bonds
Aldehyde dehydrogenase 1 family member L1 (fragment)	10	I1U3B9	OS	56692	242	9	3%	Oxidation reduction
Enolase (fragment)	11	Q9N113	OS	11952	14465	427	81%	Glycolysis
Phosphoglycerate kinase	12	B7TJ13	OS	44936	13304	446	73%	Glycolysis
Beta-actin variant 2	12	D7RIF5	OS	42052	2113	109	51%	Sarcomeric function
Enolase (fragment)	13	Q9N113	OS	11952	1974	50	79%	Glycolysis
Aldolase A (fragment)	13	Q9N116	OS	15432	1900	58	61%	Glycolysis
Aldolase A (fragment)	14	Q9N116	OS	17291	522	9	74%	Glycolysis
Glyceraldehyde-3-phosphate dehydrogenase	15	D7R7V6	OS	36110	62155	2031	78%	Glycolysis
Lactate dehydrogenase A (fragment)	16	Q9N109	OS	13391	15092	534	73%	Glycolysis
14-3-3 protein epsilon	17	P62262	OS	29326	4074	140	64%	
Superoxide dismutase	18	C8BKD6	OS	24736	2769	104	69%	Oxidation reduction
RAN	18	C5IIA0	OS	24579	1113	46	61%	GTP binding
Cytochrome c oxidase subunit 2	18	B0EXQ6	OS	26134	627	23	23%	Oxidation reduction
AK1	19	C5IIA8	OS	21750	14379	623	82%	Creatine energy Metabolism
Peroxiredoxin 2	19	C8BKCS	OS	22059	1768	76	69%	Cell redox homeostasis
Alpha-crystallin B chain	20	Q5ENY9	OS	20054	6559	341	48%	Aggregation of proteins
S-phase kinase-associated protein 1A	20	B8QGA5	OS	18817	257	7	49%	Kinase activity
Myoglobin	21	P02190	OS	17044	32908	962	97%	Oxygen transport

¹⁾Protein names and accession numbers were obtained from the UniProt database.

²⁾Band numbers of the identified proteins.

³⁾OS, Ovis aries; BOS, Bovine aries

⁴⁾Molecular mass of identified proteins (recorded in the UniProt database).

⁵⁾The MASCOT baseline significant score is 70. For the proteins identified in more than one band, the highest score was presented.

⁶⁾Number of matched peptides.

⁷⁾Percentage of coverage of the entire amino acid sequence.

Phosphorylation of sarcoplasmic proteins in response to salt To confirm that salt has no effect on the global phosphorylation level of sarcoplasmic proteins, sarcoplasmic proteins extracted from meat salted for 16 h with different concentrations of salt were subjected to SDS-PAGE and fluorescence staining with Pro-Q Diamond dye for phosphoproteins. As shown in Fig. 4A-C, no difference ($p>0.05$) in the P/T ratios among different treatments was determined, confirming that salt had no significant effect on the global phosphorylation level of sarcoplasmic proteins in meat.

However, the phosphorylation level in most protein bands was affected by salt (Fig. 4D). The phosphorylation level of eight protein (band 1, 6, 8, 10, 11, 13, 14, and 16) bands was decreased by salt,

whereas that of five bands (band 3, 7, 12, 19, and 21) was increased by salt. Although salt had no significant effect on the global phosphorylation of sarcoplasmic proteins, it affected the phosphorylation of individual proteins. The results indicated that salt also had effect on the phosphorylation of individual sarcoplasmic proteins.

Identification of phosphorylated sarcoplasmic proteins The proteins identified by LC-MS/MS represent the highly abundant proteins. The P/T ratio is a semi-quantitative method for protein phosphorylation (14,22,27). Several distinctive phosphorylated bands of sarcoplasmic proteins were selected to be identified by LC-MS/MS. Functional categories of the identified proteins were matched

manually on the basis of the retrieved information in the protein knowledgebase (UniProtKB in www.uniprot.org). According to the score, the theoretical molecular weight, number of matched peptides, and the coverage of the entire amino acid sequence (9), proteins were identified from each gel band (Table 1). In total, four categories of proteins were identified from these bands, including stress response, glycometabolism, oxidation or reduction, and others. Stress response proteins included heat shock protein alpha (HSP α) and heat shock protein 70 (HSP70). Glycometabolism proteins included glycogen phosphorylase (GP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase (ENO), AMP-activated protein kinase (AMPK), aldolase (ALD), and phosphoglycerate kinase (PGK). Oxidation or reduction proteins included superoxide dismutase (SOD) and aldehyde dehydrogenase (ALDH). Other proteins included 14-3-3 protein and myoglobin.

Salting influences the phosphorylation of glycolytic enzymes

Glycolysis, which is regulated by glycolytic enzymes, is the principal way for energy supply in postmortem muscle. Glycolysis is related to meat quality (28). The phosphorylation of many glycolytic enzymes or regulatory proteins (GP, GAPDH, ENO, AMPK, ALD, and PGK) were identified to be influenced by salting. Phosphorylation of glycolytic enzymes affected their activity and stability (14,29,30). GP was an enzyme involved in glycolysis reaction. It was activated into active enzyme form by phosphorylation, catalyzed by phosphorylase kinase, and then further promoted glycolysis (30). The phosphorylation of band 5 (GP) in the 3% salt group was higher than other groups; thus, GP had higher activity, promoted glycolysis and then regulated meat tenderness. GAPDH existed variety of post-translation modifications, whereas phosphorylation is the most common. Phosphorylation of GAPDH decreased its activity and then inhibited glycolysis (31). Finally, although protein oxidation was not analyzed, the influence of salt on the phosphorylation status of SOD, POD, and ALDH suggests that salting also impacts protein oxidation and thus meat quality.

Salting regulates the phosphorylation of heat shock proteins

Heat shock proteins (HSPs) were identified in phosphorylation proteins by LC-MS/MS, such as HSP α and HSP70. HSPs are stress response proteins and divided into multiple subfamilies. HSPs exist several post-translational modifications, which alter their functional roles. HSP α and HSP70 are essential proteins in protection against stress. HSP70 in the myocardia, liver, and soleus muscles is prone to phosphorylation following exercises (32,33). In this study, the phosphorylation of HSP α and HSP70 were detected to be reduced by salting in phosphorylation, which may alter their functional roles. HSPs impact meat tenderness (34); our study further revealed that salting might alter meat tenderness by action on the phosphorylation of HSPs.

In summary, salt influences phosphorylation and dephosphorylation of muscle proteins during salting. Salt decreases the global phosphorylation level of myofibrillar proteins and influences the

phosphorylation level of individual sarcoplasmic proteins. Our study may provide a new insight into the mechanism by which salting influences the quality of processed meat products, especially the tenderness of meat, which is related to protein degradation and muscle contraction.

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