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Phosphofructokinase mainly affects glycolysis and influences meat quality in postmortem meat

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ABSTRACT

The aim of this study was to evaluate the comparative effects of hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) on glycolysis in postmortem meat. Three specific inhibitors were respectively added to postmortem lamb to produce a HK inhibition (HKI) group, a PFK inhibition (PFKI) group and a PK inhibition (PKI) group. Meat samples that assigned to these three groups and a control (CON) group were stored at 4 °C for 5 d. The results indicated that the activity of HK, PFK and PK were effectively restrained during storage. PFK activity was significantly correlated with lactate content ($P < 0.05$), but HK and PK activity showed a weak relationship with lactate content ($P > 0.05$). The myofibrillar fragmentation index of the PFKI and PKI groups were significantly lower than that of the CON and HKI groups ($P < 0.05$). The degradation levels of troponin T and desmin were the smallest in the PFKI group. The result of the myoglobin spectrum at 540 nm and 580 nm showed a decreasing level of oxymyoglobin after HK, PFK and PK inhibition. In conclusion, PFK showed a more dominant effect on glycolysis than HK and PK in postmortem muscle, which may provide a targeted strategy for meat quality regulation through glycolysis.

1. Introduction

Glycolysis is the main way of energy metabolism in postmortem meat, which is essential for the development of meat quality. Fast glycolysis may cause PSE (pale, soft, exudative) meat but slow glycolysis may cause DFD (dark, firm, dry) meat (Adzitey & Nurul, 2011). The process of glycolysis leads to lactate accumulation which contributes to a pH decline and results in a specific ultimate pH (pH_u) in postmortem meat (Ijaz et al., 2020). Low a^* in a high pH_u (5.80–6.10) beef suggests that an insufficient glycolysis counterbalances meat color (Zhang et al., 2018). A fast pH decline causes μ-calpain activation and myofibril fragmentation in early postmortem meat (Pomponio et al., 2010). The final development of meat tenderness is related to the pH_u through myofibrillar protein degradation (Lomiwes et al., 2014).

Glycolysis is an enzymatically controlled process. The activity of glycolytic enzymes is important for glycolysis regulation. Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are known as the three glycolytic rate-limiting enzymes. HK is active in the first step of glycolysis and catalyzes glucose to glucose-6-phosphate (G6P). There are two isoforms of HK that HK I is mainly expressed in brain and HK II is mainly expressed in cardiac muscle (Pastorino & Hoek, 2008). HK regulates glycolysis and ATP through acting as a glucose sensor (Joice et al., 2012). PFK catalyzes the second step of glycolysis by the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. There are three mammal PFK isoforms identified in muscle (PFKM), liver (PFKL) and platelets (PFKP) (Kloos et al., 2015). PK is responsible for the last step of glycolysis which catalyzes phosphoenolpyruvate (PEP) to pyruvate. Four PK isoforms including PKL (in liver), PKR (PK red cell), PKM1 and PKM2 (in muscle) are found in mammals (Zheng et al., 2021). Although the three enzymes are considered as glycolytic rate-limiting enzymes, the step catalyzed by PFK is always regarded as a critical control point of glycolysis in different organisms (McGresham et al., 2014).

Oxygen supply is stopped after animals are slaughtered and the homeostasis is broken due to prolonged hypoxia. The inner environment of postmortem meat is different from that of living cells. Thus, it is possible that the roles of HK, PFK and PK in postmortem meat might be different

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compared to their roles in a living organism. The result of an *in vitro* study suggested that PFK lost its activity at pH 5.5 but phosphoglucoisomerase, aldolase, lactate dehydrogenase, pyruvate kinase remained their activity at aforementioned pH (England et al., 2014). The chilling of broiler *pectoralis major* muscle caused a high ultimate pH and less lactate accumulation via inhibition of PFK activity (Matarneh et al., 2018). Moreover, it was reported that the final pH of postmortem meat was determined by PFK activity rather than glycogen concentration (Rhoades et al., 2005). Apart from studies on the effects of PFK on postmortem meat, a number of studies focused on the role of PK. A previous study showed that PK activity was higher in PSE pork than in normal pork (Schwagele et al., 1996). However, the result of another study showed that there was no significant difference of PK activity between normal and PSE pork (Shen et al., 2006). Only a few studies investigated the HK activity in postmortem meat compared with PFK and PK. Glucose is catalyzed to G6P and then immediately participates in other energy metabolism pathways in living tissues, but the limited utilization of G6P in postmortem meat leads to its accumulation and thus inhibits HK activity (Ferguson & Gerrard, 2014). However, it is not clear to recognize which of these glycolytic rate-limiting enzymes determine glycolysis in postmortem meat.

Three inhibitors of HK, PFK and PK were respectively added to postmortem meat to compare the role of these three glycolytic rate-limiting enzymes and their effects on meat quality in this study. The results provide a comparative profile of HK, PFK and PK in postmortem meat and identifies the critical control point of postmortem glycolysis.

2. Materials and methods

2.1. Sample preparation

The *longissimus thoracis et lumborum* (LTL) muscles were collected from both sides of six Small-tailed Han lamb carcasses at Hebei Jinhong Halal Meat Co. LTD. in China. The visible fat and connective tissue were removed from these LTL muscles within 1 h after slaughtered. The LTL muscles from each carcass were then immediately minced and 20 g of the LTL muscle from each carcass was collected and defined as the sample at 0 h. Aliquots of the remaining LTL muscle from each carcass were divided into four groups: (1) adding HK inhibitor (3-bromopyruvic acid, GlpBio Technology, Shanghai, China) as the HK inhibition group (HKI); (2) adding PFK inhibitor (PFK-015, GlpBio Technology) as the PFK inhibition group (PFKI); (3) adding PK inhibitor (shikonin, GlpBio Technology) as the PK inhibition group (PKI); (4) without any inhibitor as the control group (CON). The amount of three inhibitors added was 5 mg/kg muscle samples. The samples were then stored at 4 °C for 4 h, 1 d and 5 d.

2.2. Measurement of HK, PFK and PK activity and lactate content

Approximately 0.5 g of muscle samples were separately taken from the HKI, PFKI and PKI group to measure HK, PFK and PK activity. Regarding the CON group, the activity of these three enzymes are all needed to be measured. Three commercial kits were used to detect HK, PFK and PK activity (Solarbio Life Science, Beijing, China). The changes of absorbances at 340 nm were recorded to evaluate the activity of HK, PFK and PK. The lactate content of all four groups was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.3. Protein extraction

Frozen muscle samples were ground in pre-cold lysis buffer with protease inhibitor (Epizyme Biotech, Shanghai, China). The mixture was centrifuged at 12000×*g* for 2 min. The protein supernatant was collected and then protein concentration was adjusted to 2 μg/μL with 2 × loading buffer. Boiled protein samples were stored at −80 °C for further analysis.

2.4. Glycolytic enzyme abundance and myofibrillar protein degradation

The 10% TGX stain-free gels (Bio-Rad, Hercules, CA, USA) were used to perform electrophoresis.The proteins in the gel were transferred to PVDF membranes to perform western blotting at 100 V for 90 min. The same antibodies were used as reported in previous studies (Ren et al., 2022, 2023).

2.5. Myofibrillar fragmentation index (MFI)

MFI was detected as described by Li et al. (2018). Approximately 1 g muscle sample was mixed with 10 mL pre-cold buffer A (pH 7.1) including 100 mM KCl, 20 mM K_2HPO_4, 1 mM EDTA, 1 mM $MgCl_2$ and 1 mM NaN_3 (all these chemicals came from Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China). The mixture was homogenized with an UltraTurrax Disperser S25 (IKA-Werke GmbH & Co. KG, Staufen, BW, Germany). The pellets were resolved in pre-cold buffer A after centrifugation. Protein concentration of the pellets was adjusted to 0.5 mg/mL. The absorbance at 540 nm was recorded as A540. The results of MFI were calculated as A540 × 200.

2.6. Myoglobin spectrum measurement

Frozen muscle samples were homogenized with 5 times the volumes of pre-cold phosphate buffer (40 mM, pH 6.8, Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China). The supernatant was filtered after centrifugation (Kim et al., 2013). The absorbance of filtered samples was measured from 450 nm to 650 nm through a spectrophotometer (Spark, Tecan Group Ltd., Männedorf, Zurich, Switzerland).

2.7. Statistical analysis

Data was recorded as means ± standard error and analyzed in SPSS Statistic 22.0 (SPSS Inc., Chicago, IL, USA) by a general linear model with the groups and times as fixed factors. The comparisons between different groups were analyzed by one-way analysis of variance (Duncan's multiple range test) and a *t*-test ($P < 0.05$). Principal component analysis was carried out in Origin 2021 (OriginLab Co., Northampton, Massachusetts, USA).

3. Results

3.1. The effect of glycolytic enzyme inhibitors on HK, PFK and PK activity

Three different kinds of inhibitors were added to postmortem muscle to inhibit HK, PFK and PK, respectively. There were no significant differences of HK, PFK an PK activity at 0 h between the CON group and the other groups ($P > 0.05$, Fig. 1). HK, PFK and PK activity all decreased significantly when incubating with specific inhibitors for 4 h ($P < 0.05$, Fig. 1). Compared to the CON group at 4 h, the activity of HK, PFK and PK respectively decreased 68%, 11% and 17%. HK activity increased at 4 h and then declined at 1 d and 5 d in the CON group, but it rose during the whole incubation in the HKI group (Fig. 1A). PFK activity in the PFKI group was lower than that in the CON group when incubated at 4 h and 1 d ($P < 0.05$, Fig. 1B). PFK activity was not different at 5 d between the CON and PFKI group ($P > 0.05$, Fig. 1B). PK lost 11%–17% activity in the PKI group compared that in the CON group during 4 h–5 d ($P < 0.05$, Fig. 1C). These results suggested that adding specific inhibitors indeed reduced HK, PFK and PK activity in postmortem muscle.

3.2. HK, PFK and PK abundance

At none of the storage times did the abundance of HK, PFK and PK differ between the CON group and the respective HKI, PFKI and PK group ($P > 0.05$, Fig. 2). This suggests that the addition of inhibitors did not inhibit glycolytic enzymes abundance. The abundance of HK was not

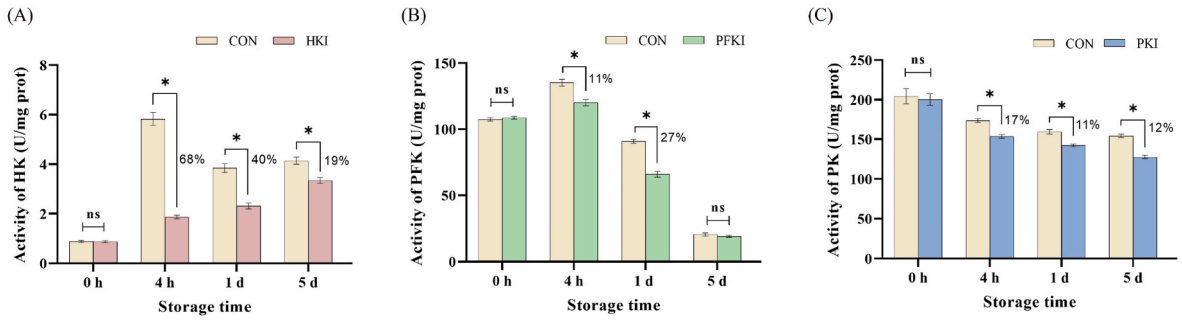

Fig. 1. The effect of three specific inhibitors on HK, PFK and PK activity, respectively. (A) HK activity in the CON and HKI group. (B) PFK activity in the CON and PFKI group. (C) PK activity in the CON and PKI group. "*" represents significant differences at the same storage time between two groups ($P < 0.05$). "ns" $P > 0.05$. HKI: hexokinase inhibitor group. PFKI: phosphofructokinase inhibitor group. PKI: pyruvate kinase inhibitor group.

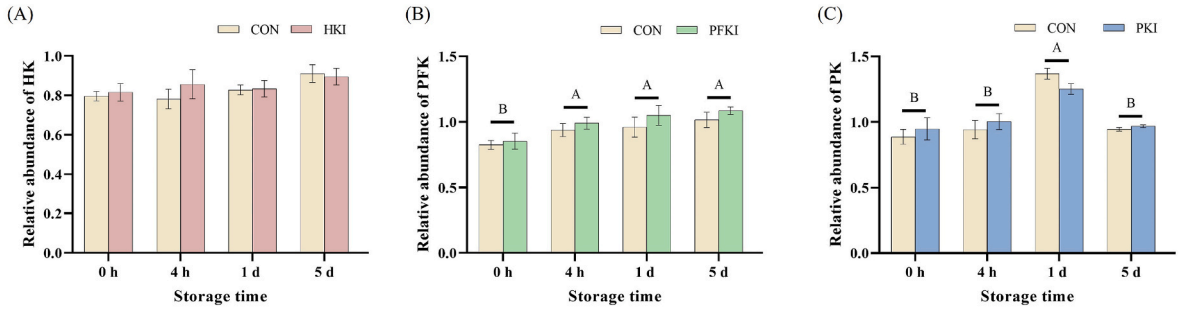

Fig. 2. The relative abundance of HK, PFK and PK during storage. (A) HK abundance in the CON and HKI group. (B) PFK abundance in the CON and PFKI group. (C) PK abundance in the CON and PKI group. A-B represents significant differences among different storage times ($P < 0.05$). HKI: hexokinase inhibitor group. PFKI: phosphofructokinase inhibitor group. PKI: pyruvate kinase inhibitor group.

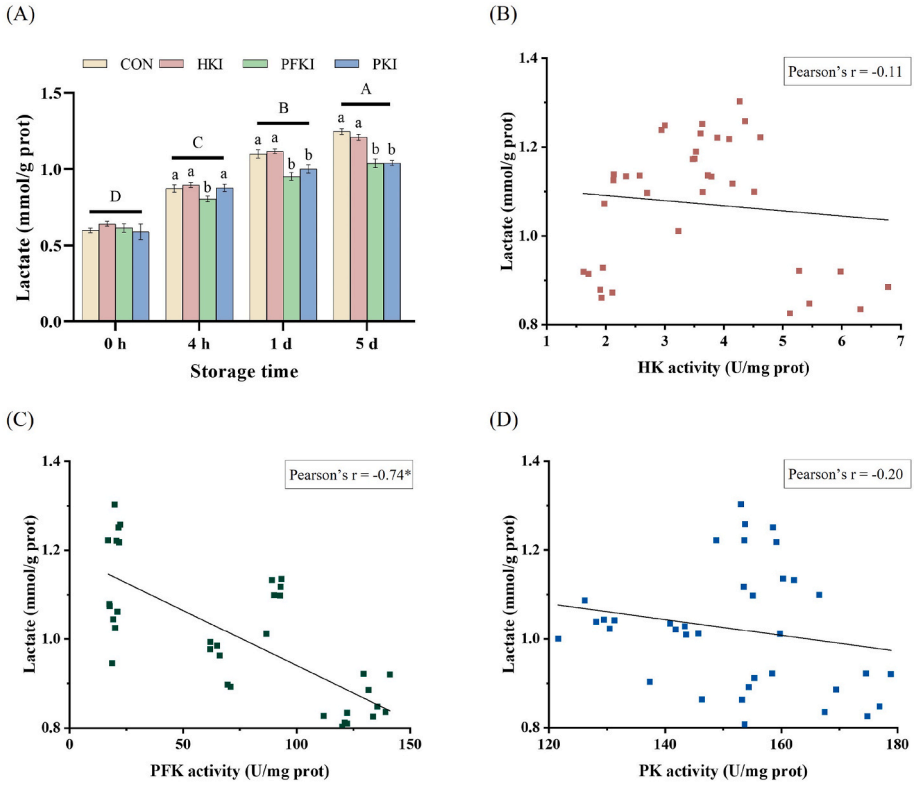

Fig. 3. The relationship of lactate and HK, PFK and PK. (A) The lactate content during storage in the four groups. a-b represents significant differences at the same storage time among the four groups ($P < 0.05$). A-D represents significant differences among different storage times ($P < 0.05$). (B) The correlation between lactate and HK activity. (C) The correlation between lactate and PFK activity. (D) The correlation between lactate and PK activity. HKI: hexokinase inhibitor group. PFKI: phosphofructokinase inhibitor group. PKI: pyruvate kinase inhibitor group.

significantly different from 0 h to 5 d storage in both the CON and HKI group ($P > 0.05$, Fig. 2A). PFK abundance at 4 h, 1 d and 5 d was higher compared with that at 0 h in both the CON and PFKI group ($P < 0.05$, Fig. 2B). The abundance of PK at 1 d was significantly higher than that at other storage times for both the CON and PKI group ($P < 0.05$, Fig. 2C).

3.3. The relationship between lactate and the activity of HK, PFK, and PK

Lactate content was measured to investigate the effect of the three glycolytic rate-limiting enzymes on glycolysis. Lactate content increased significantly from 0 h to 5 d ($P < 0.05$, Fig. 3A). Lactate content in the PFKI group was lower than that in the other three groups at 4 h ($P < 0.05$, Fig. 3A). When stored for 1 d and 5 d, the lactate content in the CON and HKI group was higher than that in the PFKI and PKI group ($P < 0.05$, Fig. 3A). This result displayed that the inhibition of PFK and PK activity might have a greater effect on glycolysis than the inhibition of HK activity. The linear fit was performed to better understand the relationship between the lactate content and the activity of the three glycolytic enzymes. The Pearson correlation coefficient between lactate and HK, PFK, PK activity was −0.11, −0.74 and −0.20, respectively (Fig. 3B, 3C, 3D). Only the Pearson's r of PFK was significant ($P < 0.05$, Fig. 3C). This result showed that PFK activity had a more substantial relationship with lactate content and glycolysis in postmortem muscle.

3.4. The physicochemical traits of meat tenderness and color

The MFI, the degradation of the myofibrillar protein and the myoglobin spectrum were measured to study the effect on meat tenderness and color after HK, PFK and PK activity were inhibited. There was an increasing trend of MFI in all four groups from 0 h to 5 d ($P < 0.05$, Fig. 4A). The MFI of the PFKI and PKI group significantly declined at 1 d and 5 d compared to the MFI of the CON and HKI group ($P < 0.05$, Fig. 4A). Similarly, the degradation of troponin T and desmin also raised from 0 h to 5 d ($P < 0.05$, Fig. 5). Troponin T degradation of the PFKI group at 1 d was significantly lower than that of the other three groups ($P < 0.05$, Fig. 5A). Desmin degradation of the PFKI group was significantly lower than that of the CON group only ($P < 0.05$, Fig. 5B). The absorbance spectrum showed the different statuses of myoglobin. The absorbance values of the four groups at 4 h were higher than those at 0 h, 1 d and 5 d (Fig. 4B). The absorbance at 540 nm and 580 nm represented the oxymyoglobin status. When stored at 4 h, the absorbance of the CON group at 540 nm and 580 nm were highest, followed by that of the HKI, PKI and PFKI group. The myoglobin absorbance decreased significantly when stored at 1 d and 5 d in all four groups.

3.5. PCA of glycolytic enzymes and meat quality traits

A PCA of HK, PFK and PK activity and other traits was performed. HK activity was mainly influenced by PC2 but other traits were mainly affected by PC1 (Fig. 6A). Therefore, it can be concluded that the HK activity and the other traits measured did not have a strong relationship. For the PCA on PFK activity, the PC1 accounted for 88.2% and the PC2 accounted for 6.1% (Fig. 6B). The PFK activity was negatively correlated with the degradation of troponin T and desmin. PK activity was greatly influenced by PC2, in contrast to the other traits (Fig. 6C). These results suggested that PFK activity might be more related to other meat quality traits when compared to HK and PK activity.

4. Discussion

HK, PFK and PK are three well-known rate-limiting enzymes in the glycolytic pathway. However, their specific roles in postmortem glycolysis have yet to be comprehensively explored. In the present study, three inhibitors were added to restrain HK, PFK and PK activity, respectively. The results showed that PFK had a greater influence on the glycolysis rate and meat quality compared with HK and PK. This is consistent with the fact that in living organisms PFK is the first regulation point of glycolysis (Currie et al., 2009). The activity of PFK is regulated by many factors such as ADP, AMP, ATP, citric acid, and fructose 2, 6-diphosphate. Moreover, the regulation of PFK is more elaborate than that of HK and PK. The pH in living organisms is always neutral, so it will not affect PFK activity largely in living tissues. However, the pH value decreases to an acidic condition in postmortem muscle. The effect of pH on PFK activity should however not be overlooked in postmortem muscle. England et al. (2014) demonstrated that PFK is responsible for glycolysis termination in postmortem muscle because other glycolytic enzymes but PFK are still active at or below pH 5.5. In addition, lactate accumulation restrains PFK activity through dissociating PFK high-active tetrameters to low-active dimers and reducing the affinity of PFK and its substrates (Costa et al., 2007). In the present study, PFK lost more than 50% activity from 0 h to 5 d, which was the highest inactivation percentage when compared with HK and PK (Fig. 1). Although PFK abundance showed a significant increase from 4 h to 5 d (Fig. 2), it did not prevent the drastic loss of PFK activity. The results demonstrated that the transformations in postmortem muscle were critical for PFK activity regulation. Thus, the higher sensitivity of PFK for pH, allosterism, and lactate content might cause the predominance of PFK in glycolysis of postmortem muscle.

PK is always regarded as a vital glycolytic enzyme in postmortem muscle. Its expression was regulated by miR-152 and significantly affected lactate, b^* and drip loss (Shen et al., 2022). The changes of PK activity seemed to have more effect on MFI and myofibrillar degradation

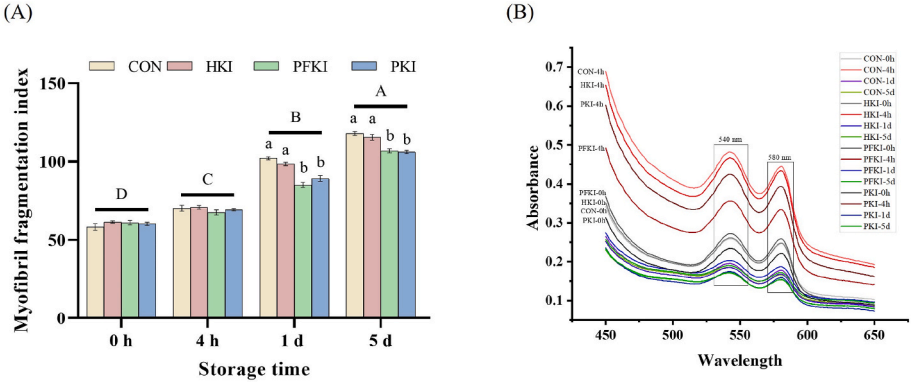

Fig. 4. Myofibril fragmentation index (A) and myoglobin spectrum (B) in the four groups during storage. a-b represents significant differences at the same storage time among the four groups ($P < 0.05$). A-D represents significant differences among different storage times ($P < 0.05$). HKI: hexokinase inhibitor group. PFKI: phosphofructokinase inhibitor group. PKI: pyruvate kinase inhibitor group. The absorbance at 540 nm and 580 nm represents the oxymyoglobin status.

(Figs. 4A and 5). There was high pyruvate content in relative low pH meat with better tenderness in a previous study result (Zhao et al., 2022), suggesting that PK activity might prompt meat tenderization. Nevertheless, the result of the present study did not show a strong relationship between PK activity and lactate (Pearson's r = −0.20, $P > 0.05$, Fig. 3D). PK activity was less decreased compared with the inhibition of HK and PFK (Fig. 1), which might be a possible reason of that. Similarly with the results of the present study, Chen et al. (2019) also reported an insignificant relationship between PK activity and lactate. Besides the role as a glycolytic enzyme, PK also processes other functions such as protein kinase. The results of our previous study showed that PK catalyzed myofibrillar protein phosphorylation and then inhibited their degradation (Ren et al., 2023). There were more than 900 substrates of PK being identified, suggesting a huge range of PK induced phosphorylation (He et al., 2016). The conversion of PK between a glycolytic enzyme and a protein kinase is an interesting issue in postmortem meat. The purified PK during different period meat is extracted in our ongoing study, which aims to investigate the relationship between the two functions of PK.

A previous study reveals that HK controls glycolytic flux in fast-growth tumor cells (Marin-Hernandez et al., 2006), and when HK is knocked out, the glycolysis process is inhibited in hepatoma cells (DeWaal et al., 2018). However, HK activity did not seem to be linked to glycolysis in postmortem muscle (Fig. 3B). HK is not as frequently examined in meat science as PFK and PK. HK catalyzes glucose to G6P in live tissues but the blood supplement is stopped after animal slaughter, which limits glucose to enter muscle cells. Glycogen transfers to glucose-1-phosphate (G1P) firstly and then phosphoglucosemutase catalyzes G1P to G6P. Thus, glycogen is the main energy source rather than glucose in postmortem muscle. The environment of postmortem muscle might limit the role of HK as a glycolytic enzyme. In addition, HK activity is suppressed when blocking its phosphorylation (Yao et al., 2018). There is no phosphorylated site of HK being identified in postmortem lamb (Chen, Li, et al., 2019), which might be another reason of the low HK activity in postmortem muscle. The role of HK in postmortem muscle is an intriguing question. HK possesses a berth sequence to mitochondria allowing HK to bind to the voltage dependent anion channel (VDAC) (Wilson, 2003). The affinity of HK for the VDAC in the mitochondrial outer membrane not only regulates energy metabolism strategy but also arranges apoptosis through repressing mitochondrial permeability (Rodriguez-Saavedra et al., 2021). Thereby, the role of HK needs to be further examined to better understand the importance of HK in postmortem muscle.

Lactate is a product of glycolysis and affects meat quality through joint effect with other endogenous factors. A previous study reported that injection of sodium lactate improved meat tenderness (Vote et al., 2000), which was consistent with the result of the present study. Another study added calcium lactate to meat, and its results showed that the calcium lactate activated *m*-calpain and thus increased the degradation of desmin and troponin T (Kim et al., 2012). Lactate accumulation leads to pH decline. The fast pH decline rate contributed to an earlier activation of μ/m calpain and subsequently caused the increased degradation of myofibrillar proteins (Huang et al., 2016). Besides meat tenderness, other parameters of meat quality also are affected by glycolysis. Lactate had a straight effect on improving meat color through increasing metmyoglobin reducing activity (Ramanathan & Mancini, 2010). The ultimate pH caused by glycolysis termination also influence meat color development. High ultimate pH contributed to an incidence of dark-cutting beef (Zhang et al., 2018). The pH value of meat with low drip loss was significantly higher than that of meat with high drip loss during 7 d display because of the possible effect of pH on protein properties (Zuo et al., 2016). The pH value was significantly different between 98 days and 120 days of Beijing You chicken, and the results of metabolomics showed the various patterns of amino acids, organic acids, carbohydrates, lipids, nucleotides, vitamin and co-enzyme factor (Ge et al., 2023). Glucose metabolism, lipid metabolism and protein metabolism coregulate the production of flavor precursors. Thus, enzymatically controlled glycolysis impacts final meat quality. Regulating the critical control point of glycolysis is an effective approach to develop meat quality.

5. Conclusion

Three types of inhibitors were added to postmortem muscle to evaluate the effect of HK, PFK and PK on glycolysis and meat quality, respectively. PFK displayed a more prominent role in the regulation of glycolysis, myofibrillar protein degradation and myoglobin status than HK and PK. PFK is also identified as the critical control point among three glycolytic rate-limiting enzymes in postmortem muscle. This comparative study of HK, PFK and PK provides a better understanding of postmortem glycolysis.

CRediT authorship contribution statement

Chi Ren: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Yuqiang Bai:** Investigation. **Martine Schroyen:** Writing – review & editing, Supervision. **Chengli Hou:** Writing – review & editing. **Xin Li:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Zhenyu Wang:** Writing – review & editing. **Dequan Zhang:** Writing – review & editing, Supervision, Data curation, Conceptualization.

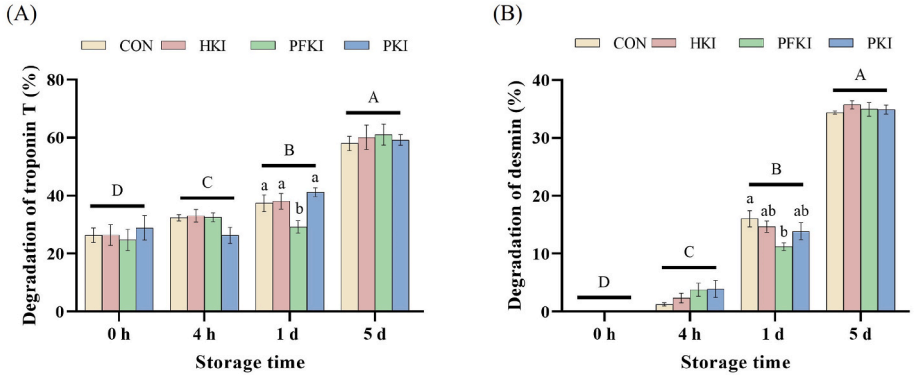

Fig. 5. The degradation of troponin T (A) and desmin (B) in the four groups during storage. a-b represents significant differences at the same storage time among the four groups ($P < 0.05$). A-D represents significant differences among different storage times ($P < 0.05$). HKI: hexokinase inhibitor group. PFKI: phosphofructokinase inhibitor group. PKI: pyruvate kinase inhibitor group.

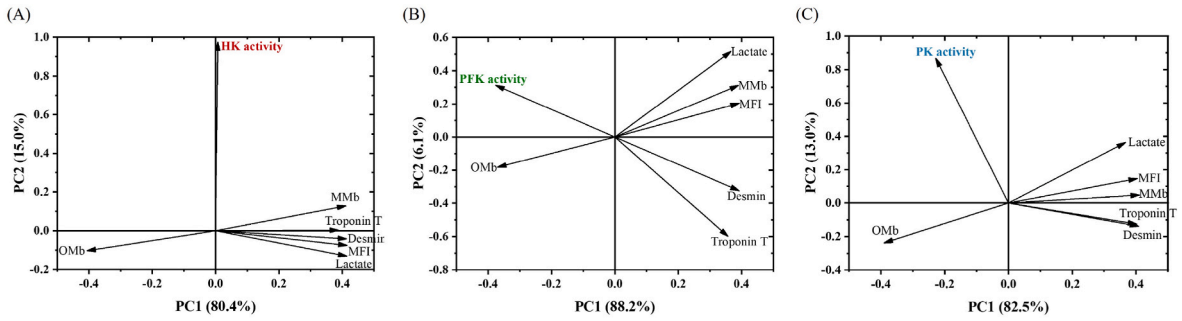

Fig. 6. Principal component analysis of HK (A), PFK (B), PK (C) and meat quality traits.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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