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The effect of protein phosphorylation and acetylation on phosphofructokinase in lamb

Chi Ren [a,b], Xinran Zhao [a], Martine Schroyen [b], Xin Li [a,*], Chengli Hou [a], Zhenyu Wang [a], Dequan Zhang [a]

[a] Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences/ Key Laboratory of Agro-products Quality & Safety in Harvest, Storage, Transportation, Management and Control, Ministry of Agriculture and Rural Affairs, Beijing, 100193, PR China
[b] Precision Livestock and Nutrition Unit, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés 2, Gembloux, 5030, Belgium

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ABSTRACT

Phosphofructokinase (PFK) is a key glycolytic rate-limiting enzyme that affects final meat quality through glycolysis regulation. PFK occurs protein phosphorylation and acetylation in postmortem meat, but how they affect PFK activity has not been well studied. Phosphorylation and acetylation of PFK were adjusted in an in *vitro* system by adding protein kinase A (PKA) and P300/CBP-associated factor (PCAF), respectively. PFK activity significantly decreased after increasing its phosphorylation level by PKA ($P < 0.05$). PCAF increased PFK acetylation level, but did not significantly change its activity ($P > 0.05$). Five PFK variants including wtPFK (wild type), PFK_T704D (T704 phosphorylation), PFK_T704A (T704 dephosphorylation), PFK_K678Q (K678 acetylation) and PFK_K678R (K678 acetylation) were constructed to investigated the effect of T704 and K678 on enzyme activity. Kinetic analysis showed a higher K_m, lower V_{max}, and lower catalytic efficiency of PFK_T704D on fructose 6-phosphate (F6P), suggesting that T704 phosphorylation decreased PFK activity, possibly by weakening the affinity between F6P and PFK. The changes of PFK variants on ATP were slightly lower than those of PFK variants on F6P. The present study identifies T704 as a possibly vital site for PFK activity regulation and provides theoretical support for the development of meat quality preservation technology.

1. Introduction

Glycolysis is the main way of energy metabolism in postmortem meat, which is a continuous process catalyzed by ten enzymes. Among these enzymes, phosphofructokinase (PFK) is one of the three rate-limiting enzymes and is regarded as a critical control point of glycolysis (Currie et al., 2009). PFK catalyzes the conversion of fructose 6-phosphate (F6P) to fructose 1,6-bisphosphate (F16P), which is affected by allosteric regulation. ATP, F16P, fructose 2,6-bisphosphate (F26P), and citrate are allosteric activators or inhibitors of PFK (Kemp & Gunasekera, 2002). PFK loses its activity when the pH declines to 5.5, while other glycolytic enzymes are still active, which arrests the glycolysis process in postmortem meat (England et al., 2014). The PFK gene is significantly linked to pork meat color, water holding capacity and marbling (Wang et al., 2014). A previous study reported that PFK activity was negatively correlated with fluid loss (Allison et al., 2003). Antemortem stress causes deterioration of meat quality with high PFK activity (Sun et al., 2019). In addition, PFK abundance is higher in normal meat than in pale, soft, and exudative-like (PSE-like) meat, which is negatively related to L^* and b^* (Desai et al., 2016). Thus, PFK is an important glycolytic enzyme involved in the regulation of the final meat quality.

Previous studies have revealed that protein post-translational modifications (PTMs) greatly affect meat quality development (Li et al., 2021). Glycolytic enzymes are among the largest clusters of PTMs in postmortem meat. The activity of glycolytic enzymes is affected by PTMs, which influences the glycolysis of postmortem meat. Phosphorylation of PFK is significantly related to the pH decline in lamb meat (Chen et al., 2018). When phosphatase inhibitor was added to increase PFK phosphorylation level, its activity increased during incubation for 6–12 h at 4 °C (Chen et al., 2019). Phosphorylation of PFK S667 was decreased in low-quality caprine meat, which might inhibit PFK activity (Liu et al., 2018). The result of a previous study showed that the increasing phosphorylation level of PFK leaded to the occurrence of PSE meat (Shen et al., 2006). In addition to the effects of protein phosphorylation on PFK, some studies have focused on protein acetylation.

* Corresponding author.
E-mail address: xinli.caas@gmail.com (X. Li).

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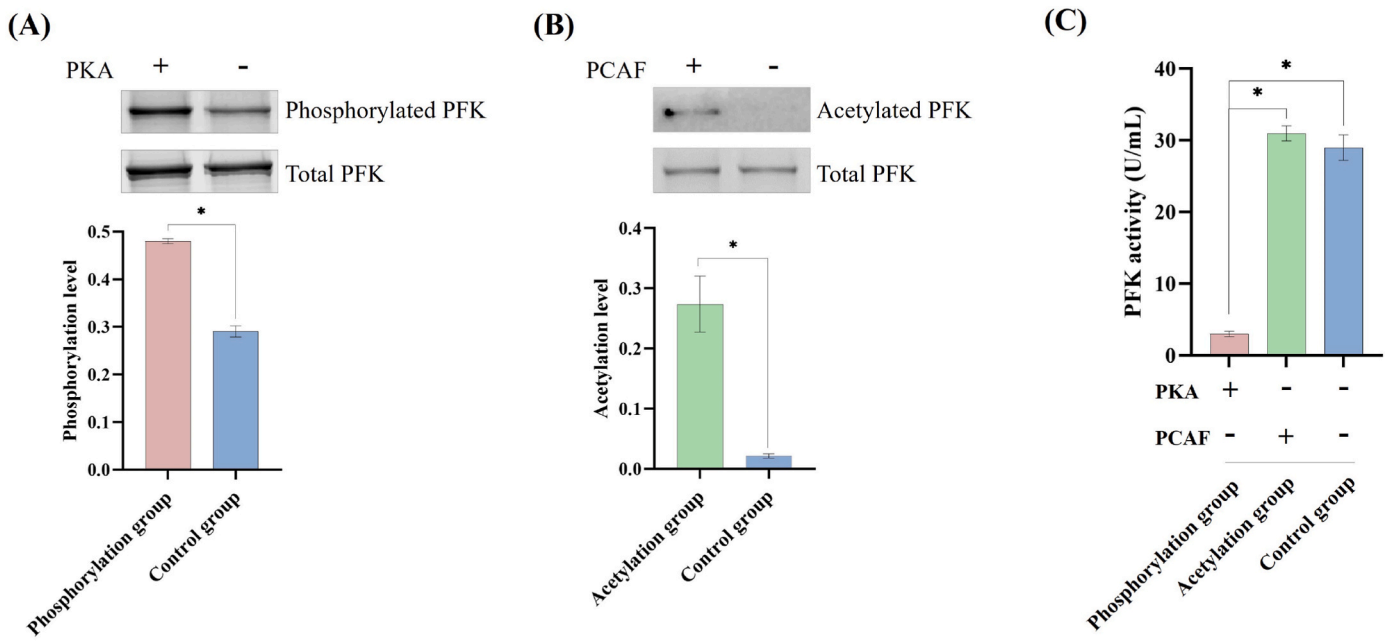

Fig. 1. The effect of phosphorylation and acetylation on PFK activity in *vitro*. (A) The comparison of PFK phosphorylation level between the phosphorylation and control group. (B) The comparison of PFK acetylation level between the acetylation and control group. (C) The comparison of PFK activity among the phosphorylation, acetylation and control group. PKA and PCAF were used to change PFK phosphorylation and acetylation levels. $*P < 0.05$.

Six acetylated sites of PFK have been identified in porcine meat, and the acetylation level of PFK is upregulated in stressed meat (Jiang et al., 2019; Zhou et al., 2019). The higher PFK acetylation level in postmortem 1 d led to a slower pH decline, which caused the tough tenderness (Ren et al., 2022). PFK acetylation seems to be correlated with meat quality, but the effect of protein acetylation on PFK activity in postmortem meat has not been evaluated.

Many phosphorylated and acetylated sites of PFK have been identified in meat samples, but the specific effects of these sites have not yet been investigated. The results of studies in life science field have revealed that PFK function is regulated by phosphorylation and acetylation. Insulin controlled glycolysis by phosphorylating PFK at S775 (Yugi et al., 2014). When S775 was mutated to S775D or S775E to mimic phosphorylation, PFK showed higher activity than the wild PFK. Acetylation of K395 induced PFK transfer to plasma membrane and subsequently promoted the binding of PFK to epidermal growth factor receptor (Lee et al., 2018). Thus, it is necessary to determine the roles of phosphorylated and acetylated sites in meat PFK. The objective of the present study was to investigate the effect of specific phosphorylation and acetylation sites on lamb PFK activity and elucidate the possible mechanism.

2. Material and methods

2.1. PFK phosphorylation and acetylation in vitro

Protein kinase A (PKA) is a kind of kinase to catalyze PFK phosphorylation (Costa Leite et al., 2007), and PCAF (P300/CBP-associated factor) is a kind of acetyltransferase to catalyze PFK acetylation (Li, Li, et al., 2018). In order to investigate whether PFK was phosphorylated or acetylated by them, a *vitro* system was performed. Three groups were used to regulate PFK phosphorylation and acetylation level: 1) PKA and ATP were added to increase PFK phosphorylation level (PFK phosphorylation group); 2) PCAF and CoA were added to increase PFK acetylation level (PFK acetylation group); and 3) without adding any PTM regulator (control group). They were incubated in a buffer containing 4 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT at 37 °C for 1 h. Part of the sample was collected for PFK activity analysis using a commercial kit (Solarbio Life Science, Beijing, China).

2.2. PFK expression and purification in E. coli

The Total RNA was extracted from the *longissimus thoracis lumborum* muscle of small-tailed Han lamb carcass at Beijing Ershang Meat Food Group Co., LTD in China. A reverse transcription kit (TaKaRa, Dalian, China) was used to obtain cDNA. The forward primer 5′-GTGACCCATGAAGAGCACC and reverse primer 5′-CTAGATGTTAGCTTCTCCGGACC were designed to obtain the full-length PFK. PFK gene was cloned into the pET-32a vector (Solarbio Life Science, Beijing, China) and the recombinant plasmid was transformed into *E. coli* BL21 (DE3) (TransGen Biotech Co., Ltd, Beijing, China). The recombinant plasmid of wild type PFK (wtPFK) was used as the template to produce mutated PFK, including T704D, T704A, K678Q and K678R, by site-directed mutagenesis. The expression and purification of PFK were performed as previously described with some modification (Currie et al., 2009; Li, Li, et al., 2018). A single colony was cultured in Luria-Bertani medium with 50 μg/mL ampicillin at 37 °C until the OD600 reached 0.6, and then cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 6 h. The cells were harvested and proteins were eluted in a Ni-NTA column with PBS-NaCl, 500 mM imidazole, pH 7.4. PKM variants were purified using Superdex 200 (GE, Boston MA, USA).

2.3. Enzyme kinetics assays

A coupled reaction system was used to determine PFK kinetics with different concentrations of ATP and F6P (Bruser et al., 2012) at 37 °C for 30 min. The wild and mutated PFK were mixed with 50 mM HEPES, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM NADH, 0.45 units/ml aldolase, 4.5 units/ml triosephosphate isomerase, and 1.5 units/ml glycerol phosphate dehydrogenase, pH 7.0. The concentrations of ATP and F6P were set at 0.5, 1, 1.5 and 2 mM. Absorbance was recorded at 340 nm.

2.4. Molecular simulation

2.4.1. Molecular dynamics simulation of the wild and mutated PFK

The original 3D structure of PFK was constructed using the BIOVIA

Table 1
The differences of total energy between PFK phosphorylation and dephosphorylation after molecular dynamics simulation.

Phosphorylation		Dephosphorylation		Difference[|a-b|]
Mutation	Total energy (kcal/mol)[a]	Mutation	Total energy (kcal/mol)[b]	
T193D	−167434.70	T193A	−167396.92	37.78
T194D	−167425.12	T194A	−167501.69	76.57
S377D	−167199.90	S377A	−167125.15	74.75
S454D	−167340.54	S454A	−167372.46	31.92
Y455E	−167660.36	Y455F	−167810.97	150.61
T486D	−167563.89	T486A	−167413.62	150.28
S667D	−167762.34	S667A	−167512.74	249.60
T704D	−166987.97	T704A	−167492.19	504.22

|a-b|: The absolute value of total energy difference between phosphorylation mutation and dephosphorylation mutation.
[a] The total energy of phosphorylation mutation.
[b] The total energy of dephosphorylation mutation.

Discovery Studio 2019 (Dassault Systemes BIOVIA Ltd., France). Eight phosphorylated sites (T193, T194, S377, S454, Y455, T486, S667, T704) and ten acetylated sites (K107, K141, K280, K395, K476, K615, K656, K678, K744, K754) of PFK was respectively mutated according to the phosphoproteomic and acetylomic analysis in lamb (Ren, Bai, Zhang, et al., 2024). The total energy of the mutated PFK was calculated to evaluate the effect of specific PTM sites on PFK stability.

2.4.2. PFK docked to F6P and ATP

The 2D structures of F6P (PubChem CID: 69507) and ATP (PubChem SID: 24770838) were downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/). PFK was regarded as a receptor, F6P and ATP were regarded as ligands. Molecular docking was performed using AutoDock Vina v1.1.2. A higher relative value of vina affinity indicated a stronger binding between receptor and ligand. The 2D interaction picture was analyzed in BIOVIA Discovery Studio 2019 and the 3D interaction picture was analyzed in PyMOL 2.5.4.

2.5. Statistical analysis

The data were recorded as mean values ± standard error and analyzed in SPSS Statistic 22.0 (SPSS Inc., Chicago, IL, USA) with Duncan's test. Statistical significance was set at $P < 0.05$. The phosphorylation and acetylation levels of PFK were analyzed in Quantity-One 4.6.2 (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. The effect of PFK phosphorylation and acetylation on PFK activity (in vitro)

An in *vitro* experiment was performed to investigate whether PFK phosphorylation and acetylation affected its activity. PKA and PCAF were added to the in *vitro* system to adjust PFK phosphorylation and acetylation level, respectively. The results showed that PKA and PCAF significantly increased PFK phosphorylation and acetylation levels compared to those in the control group (Fig. 1A and B, $P < 0.05$). Next, the effect of PFK phosphorylation and acetylation on PFK activity was evaluated. PFK activity was significantly inhibited in the PFK phosphorylation group (Fig. 1C, $P < 0.05$), but PFK activity in the acetylation group was not markedly different from that in the control group (Fig. 1C, $P > 0.05$). The results of the in *vitro* study showed that PFK phosphorylation induced by PKA might change its activity.

3.2. Total energy of PFK variants after molecular dynamics simulation

Total energy means the stability of a protein. We calculated the changes of total energy to screen which site had the greatest influence on PFK stability. The mutations of eight phosphorylated sites and ten acetylated sites of PFK were simulated in Discovery Studio to screen the practical mutated sites. The lower the absolute value of the total energy, the more stable is the protein. The difference between PFK_T704D and PFK_T704A was the highest among the eight phosphorylated sites, suggesting that phosphorylation and dephosphorylation of PFK_T704 might have the greatest effect on PFK (Table 1). Similarly, the difference in PFK_K678Q and PFK_K678R was the highest among the ten acetylated sites, suggesting that acetylation and deacetylation of PFK_K678 might have the greatest effect on PFK (Table 2). Thus, PFK_T704 and PFK_K678 were selected as practical mutated sites in the next study.

Table 2
The differences of total energy between PFK acetylation and deacetylation after molecular dynamics simulation.

Acetylation		Deacetylation		Difference[|a-b|]
Mutation	Total energy (kcal/mol)[a]	Mutation	Total energy (kcal/mol)[b]	
K107Q	−167697.76	K107R	−167451.29	246.46
K141Q	−167889.42	K141R	−167709.35	180.07
K280Q	−167525.26	K280R	−167447.10	78.16
K395Q	−167634.92	K395R	−167519.55	115.37
K476Q	−167361.96	K476R	−167559.35	197.39
K615Q	−167539.07	K615R	−167812.39	273.32
K656Q	−167394.40	K656R	−167378.17	16.23
K678Q	−167337.25	K678R	−167671.42	334.17
K744Q	−167925.03	K744R	−167688.74	236.29
K754Q	−167478.57	K754R	−167760.88	282.31

|a-b|: The absolute value of total energy difference between acetylation mutation and deacetylation mutation.
[a] The total energy of acetylation mutation.
[b] The total energy of deacetylation mutation.

Table 3
Kinetic parameters of PFK variants on different substrates (F6P and ATP).

PFK variants	Substrates	K_m (mM), V_{max} (μM/min), V_{max}/K_m (h^{-1})
Wild type PFK	F6P	1.22, 41.54, 2.04
	ATP	3.13, 46.52, 0.89
PFK_T704D	F6P	1.79, 32.05, 1.08
	ATP	3.26, 43.27, 0.80
PFK_T704A	F6P	1.21, 41.72, 2.07
	ATP	3.00, 46.18, 0.92
PFK_K678Q	F6P	1.12, 40.28, 2.16
	ATP	3.15, 47.29, 0.90
PFK_K678R	F6P	1.33, 45.17, 2.04
	ATP	3.01, 46.44, 0.92

3.3. Kinetics analysis of PFK variants

F6P and ATP are two substrates of PFK, thus the different kinetic properties of the PFK variants were evaluated to investigate the effect of T704 and K678 mutations on PFK activity (Table 3). V_{max} represents the maximum rate of enzyme reaction. When the V_{max} of F6P was determined, the V_{max} of PFK_T704D was lower than that of other PFK variants, suggesting a decreased affinity for F6P upon PFK_T704 phosphorylation. V_{max} of PFK_K678R reached to 45.17 μM/min. The V_{max} of ATP with PFK variants was approximately 43.27–47.29 μM/min. K_m represents the substrate concentration of 1/2 V_{max}. A lower K_m value indicates a stronger affinity between the enzyme and the target substrate. The K_m of all PFK variants with ATP was higher than that with F6P, indicating that the affinity of PFK for F6P was stronger than that of PFK for ATP. The ratio of V_{max}/K_m represents the catalytic efficiency of PFK for F6P and ATP. The V_{max}/K_m of all PFK variants with F6P was higher (1.08–2.16 h^{-1}) than that with ATP (0.80–0.92 h^{-1}), which showed a higher catalytic efficiency between PFK and F6P. These results

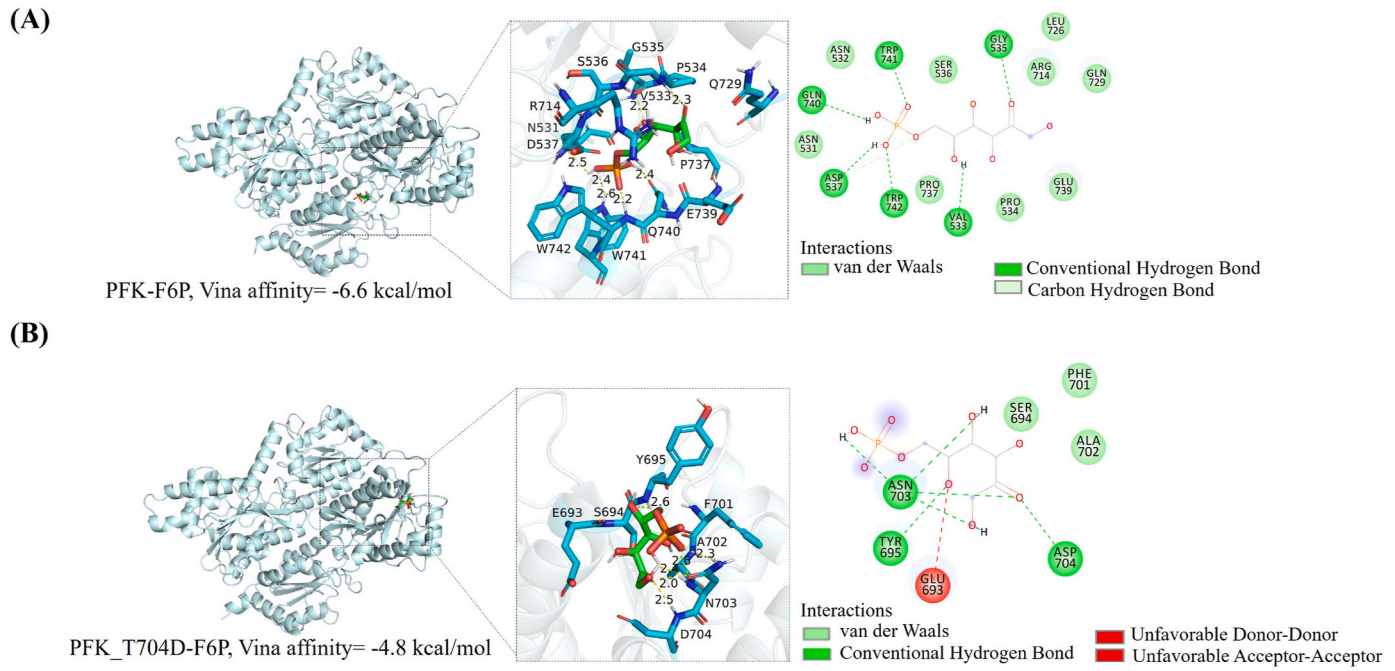

Fig. 2. Visualized docking between PFK variants and F6P. (A) Molecular docking between wtPFK and F6P. (C) Molecular docking between PFK_T704D and F6P. Vina affinity was calculated to evaluate the binding energy of PFK variants and F6P. 2D and 3D interaction patterns showed the detailed information between their binding.

indicated that T704 phosphorylation might decrease PFK activity, possibly through the weakened affinity between PFK and F6P.

3.4. *The affinity analysis by molecular docking*

Kinetic analysis showed a dramatically decreasing affinity between F6P and PFK_T704 (Table 1), which was subsequently used to visualize the affinity changes between F6P with wtPFK and PFK_T704D, respectively (Fig. 2). The vina affinity between F6P and PFK_T704D is 27% lower than that between F6P and wtPFK. The results of the molecular docking analysis were consistent with those of the kinetics analysis. Fifteen amino acids of wtPFK interacted with F6P, but only 7 amino acids of PFK_T704D interacted with F6P. The phosphorylation of T704 might decrease the targeting of F6P to PFK.

4. Discussion

PFK is one of the three glycolytic rate-limiting enzymes and is regarded as the key control point of glycolysis. The property of PFK is important for the development of meat quality through glycolysis regulation (Ren, Bai, Zhang, et al., 2024). The expression of PFK gene is related to pork color, marbling, intramuscular fat and water holding capacity (Wang et al., 2014). PFK is sensitive to temperature and pH changes in postmortem meat. Chilling leads to the pre-termination of postmortem glycolysis by inhibiting PFK activity (Matarneh et al., 2018). Postmortem glycogenolysis and glycolysis are normal within pH 6.0–7.0, but when pH decreases to 5.5, lactate accumulation is stopped due to complete inactivation of PFK (England et al., 2014). Thus, controlling PFK activity is an important strategy for regulating meat quality. PTM is a well-known process that regulates protein stability, cellular localization and function. PFK was identified as a significantly different phosphorylated and acetylated protein in lamb (Li, Li, et al., 2018), pork (Jiang et al., 2019), beef (Li et al., 2023) and chicken (Weng et al., 2022). However, the effects of phosphorylation and acetylation on PFK activity have not yet been clearly illustrated. We constructed five PFK variants to investigate the effects of phosphorylation and acetylation on PFK activity in this study.

An in *vitro* system was established to determine whether phosphorylation and acetylation affected PFK activity. PKA and PCAF are two enzymes that catalyze protein phosphorylation and acetylation (Liu et al., 1999). The results showed that phosphorylation induced by PKA decreased PFK activity. IκB kinase β (IKKβ) is a kind of protein kinase that phosphorylates PFK. A previous study showed that PFK activity decreased when phosphorylated by IKKβ (Reid et al., 2016), which was consistent with the results of the present study. Although these results displayed a decreasing activity after PFK phosphorylation, the specific phosphorylated sites were different between them. IKKβ phosphorylated PFK_S269, but in the present study, PFK_T704 phosphorylation was a concern. In another study, the relationship between PFK phosphorylation and its activity changed in the opposite manner. Protein phosphatase 4 regulatory subunit 1 (PP4R1) dephosphorylated PFK and then downregulated its activity (Park & Lee, 2023). It is limited to depict a more detailed discussion because this study did not identify the site that was dephosphorylated by PP4R1. With regard to protein acetylation, the present study showed an insignificant effect of protein acetylation on PFK activity. Referring to a previous study, PFK_K472 acetylation first changed its subcellular localization and then enhanced its sensitivity to phosphorylation and activation induced by energy stress (Li, Li, et al., 2018). Moreover, PFK_K394 deacetylation induced by sirt 2 made it more easily ubiquitinated and degraded (Gandhirajan et al., 2022). It can be concluded that PFK activity is regulated by more than one site. Confirming the effect of targeted sites on PFK activity is important to provide theoretical support for precise meat quality preservation technology. For example, PTM levels of glycolytic enzymes in postmortem meat are changed by different chilling rates (Bai et al., 2023). We can monitor the changes of PFK_T704 under different chilling rates, and then set up a specific chilling rate to regulate PFK activity and meat quality. Here, we only mutated two real sites of PFK according to the changes of total energy and investigated their effects. According to the results of sequence alignment (Fig. S1), K107, K141, T193, T194, K280, S377, K476, K615, K656, K678, K744 and K754 were fully conserved among the sequences of lamb, bovine, pig, chick and duck. These conserved residues are worth to be performed further study. The results of the present study showed that T704 was an important site for PFK activity.

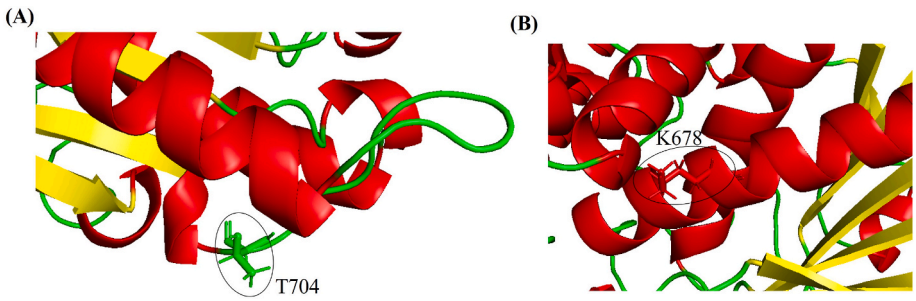

Fig. 3. The location of PFK_T704 (A) and PFK_K678 (B) in 3D structure. Yellow: sheet; red: helix; green: loop. Sheet and helix are ordered region, loop are disordered region.

Combining the results of sequence alignment, T704 might be also important in pig, chick and duck.

F6P and ATP are the two substrates for PFK to produce F16P and ADP, respectively. The binding between substrates and PFK is closely related to its activity. Kinetic analysis was performed to compare the parameters of different substrate concentrations of the PFK variants. The K_m of ATP with all PFK variants was higher than that of F6P, which displayed a lower affinity between ATP and PFK compared that between F6P and PFK. When PFK_T704 was phosphorylated, its catalytic efficiency with F6P decreased, suggesting a lower glycolytic activity of PFK_T704D. Molecular docking results also showed that PFK_T704D reduced the relevant residues involved in F6P binding. The property of the binding residues between PFK and its substrates are important for enzyme activity. After PFK_D17 was mutated to PFK_A17 to mimic dephosphorylation, a significant effect on F6P binding through K158 was observed (Currie et al., 2009). The phosphorylation mutation of PFK_T704 might change the hydrogen bonds and rotation of the side chain to affect the binding of F6P and activity sites. A previous study reported that PFK_S269 phosphorylation reduced its activity by 80% (Reid et al., 2016). S269 is located in a highly disordered region which is critical for PFK enzyme function. The disordered region is important for protein structure and function. In the present study, PFK_T704 was in a disordered region, and K678 was in a helical region (Fig. 3). This might be one of the reasons for the greater effect of the change in T704 on PFK activity than K678.

5. Conclusion

PKA-induced phosphorylation of PFK decreased its glycolytic activity. The mutations of T704 and K678 on PFK caused large differences on its total energy. Phosphorylation of PFK_T704 decreased its catalytic efficiency by reducing the affinity between F6P and PFK. The change in PFK_T704 had a greater effect on PFK activity than PFK_K678. The PFK_T704 is potentially an important site for the regulation of PFK activity and postmortem glycolysis.

CRediT authorship contribution statement

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

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.105929.

Data availability

Data will be made available on request.

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