Study on the ZnPP formation mechanism in two different optimum pH at 4.75 and 5.5 in pork

(異なる2 つの至適pH 4.75 と5.5 における豚肉の ZnPP 形成機構に関する研究)

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Abstract

Zinc protoporphyrin IX (ZnPP) is a bright red pigment formed in meat products without nitrate or nitrite. The two optimal pH values for ZnPP formation were 4.75 and 5.5. Moreover, the amount of ZnPP formed at pH 4.75 was much higher than that at pH 5.5 in pork. The number or kinds, of precursors or contributors for ZnPP formation at pH 4.75 might be different from that at pH 5.5. Although it is assumed that ferrochelatase (FECH), myoglobin as a heme donor and Zn ion play important roles in ZnPP formation, the exact mechanism is indistinct. It was reported that ZnPP formation is strongly influenced by protoporphyrin IX (PPIX) formation at pH 5.5. However, further investigations was hardly examined. Hence, it was hypothesized that ZnPP formation depends on the formation of PPIX at pH 4.75, multiple components in pork homogenate are essential to the formation of ZnPP and PPIX at pH 4.75 and 5.5, and the contributors and precursors are different between both pH conditions. Therefore, the purpose of this research was to examine ZnPP formation mechanism at optimum pH 4.75 and 5.5.

The examined various factors on PPIX formation at pH 4.75 almost coincided with those of ZnPP, suggesting that ZnPP formation at pH 4.75 was strongly affected by PPIX formation. When pork homogenate was separated into the insoluble and two water-soluble fractions (>10 and <10 kDa) by centrifugation and ultrafiltration, ZnPP and PPIX formation were suppressed at pH 4.75 in the absence of one of those fractions. However, ZnPP and PPIX formation were rescued after mixing of all three fractions. Then, heating of the <10 kDa soluble fraction did not suppress ZnPP and PPIX formation as opposed to heating of the >10 kDa soluble fraction, suggesting that protein(s) presents in the >10 kDa and heat-stable component present in the <10 kDa

soluble fraction contributed to ZnPP and PPIX formation. Furthermore, 10-30 kDa soluble fraction separated by ultrafiltration was important for ZnPP and PPIX formation at pH 4.75. Exogenous myoglobin assisted ZnPP and PPIX formation at pH 4.75. A gel filtration study showed that ZnPP and PPIX formation were significantly correlated with the endogenous myoglobin contents in the separated fractions at pH 4.75. Based on these results and previous reports, it was suggested that ZnPP is formed from myoglobin-derived heme via PPIX, and FECH from insoluble fraction, and heat-stable <10 kDa soluble component contribute to PPIX and ZnPP formation at pH 4.75 in pork.

The study on ZnPP formation mechanism at pH 5.5 also revealed that three or more components i.e. the insoluble fraction, the >10 kDa and <10 kDa water-soluble fraction are also essential for ZnPP and PPIX formation at pH 5.5 in pork. Water-soluble protein(s) with higher molecular weight than that of myoglobin was involved in ZnPP and PPIX formation at pH 5.5. Water-soluble 42 and/or 60 kDa proteins separated by ion exchange chromatography are suggested to contribute for ZnPP formation at pH 5.5. Heat-stable components in the <10 kDa soluble fraction contribute to ZnPP formation at pH 5.5. Based on these results and previous reports, it was suggested that ZnPP is formed from PPIX with the contribution of 42 and/or 60 kDa soluble protein, and FECH from insoluble fraction, 10-30 kDa soluble protein and heat-stable <10 kDa component contribute to PPIX and ZnPP formation at pH 5.5 in pork.

The present study revealed that the precursors, the contributors, and optimum temperature are different for ZnPP formation in the optimum pH at 4.75 and 5.5, thus the mechanisms are different. Although it is not clear how these two mechanisms contributed to the color formation in meat products, these findings will be helpful to elucidate the whole ZnPP formation mechanisms in meat products.

1. Introduction

The color of meat and meat products are important determinants of quality and an imperative selection criterion by consumers. The characteristic red color of cured meat products is typically due to nitrosylmyoglobin, which is formed by the reaction of nitrate/nitrite with myoglobin in the presence of endogenous or added reductants. However, the use of nitrate/nitrite is controversial to the involvement in the formation of carcinogenic N-nitrosamines (De Mey, 2014). N-nitrosamines generated from residual nitrite in cured meat products and biogenic amines after consumption have harmful health effects (Skibsted, 2011). Reduction of added nitrite is already a matter of awareness for a few decades, whereby color formation in meat products without the use of nitrate/nitrite or other artificial coloring agents is one of the challenges.

Wakamatsu et al. (2004a) identified zinc protoporphyrin IX (ZnPP) as the main pigment in Italian dry-cured Parma ham (*Prosciutto di Parma*). The content of ZnPP increases throughout the processing and maturation of Parma ham (Adamsen et al., 2006b). The amount of ZnPP in Parma ham was larger than that of heme, and ZnPP was considered for 60–70% of all porphyrins (Wakamatsu et al., 2009a; Bou et al., 2018). Later, it was reported that ZnPP can be formed in Parma-like dry-cured ham without the addition of nitrate and nitrite (Wakamatsu et al., 2009b). It was also reported that nitric oxide (NO) produced from nitrite inhibited the formation of ZnPP (Wakamatsu et al., 2010). ZnPP formation was also confirmed in several other studies focusing on production of nitrite-free meat products, such as dry-cured Iberian hams and dry-fermented sausages (Adamsen et al., 2006a; De Maere et al., 2016). Moreover, the distribution of ZnPP in Parma ham was shown by using autofluorescence (Wakamatsu et al., 2006). It was found by fluorescent analysis and ESI-MS and TOF-MS analysis

that the red pigment was present not only in Parma ham but also in Iberian ham although the pigment content in meat products cured with nitrite was very low (Adamsen et al., 2006a; Møller, et al., 2007). It was suggested to use the immediate increase of ZnPP fluorescence after slaughtering as a quality indicator of fresh pork with regard to inadequate conditioning, e.g. during transportation and/or storage (Schneider et al., 2008; Durek et al., 2012). Therefore, there is significant interest in clarifying the mechanisms of ZnPP formation in meat products, since it can play an important role in coloration without requiring the addition of nitrite or nitrate.

ZnPP is formed as a normal metabolite in trace amounts during heme biosynthesis in the living body (Labbé et al., 1999) (Fig. 1A). The heme biosynthesis pathway is initiated by the synthesis of aminolevulinic acid (ALA) from the glycine and succinyl-CoA by the enzyme ALA synthetase in mitochondria (Franken et al., 2011). The ALA is converted to porphobilinogen by ALA dehydratase in the cytoplasm. Subsequently, it transformed into coproporphyrinogen III via several enzymatic reactions in the cytoplasm. Then, it is converted into protoporphyrinogen III and protoporphyrin IX (PPIX) in mitochondria by the enzyme coproporphyrinogen III oxidase and protoporphyrinogen III, respectively. The final reaction in the biosynthetic pathway of heme is the chelation of divalent iron with PPIX by the enzyme ferrochelatase (FECH) (Elrod et al., 1997; Moretti et al., 2000). Instead of incorporating a ferrous ion to form heme; PPIX, rarely incorporates a zinc ion and ZnPP is formed (Fig. 1B). During periods of iron insufficiency or impaired iron utilization in the heme biosynthesis pathway, zinc becomes an alternative metal substrate to increase the formation of ZnPP in the living body (Labbé et al., 1999). It was also reported that the reaction of PPIX with zinc is linked and occurs as a by-product of heme biosynthesis during states of

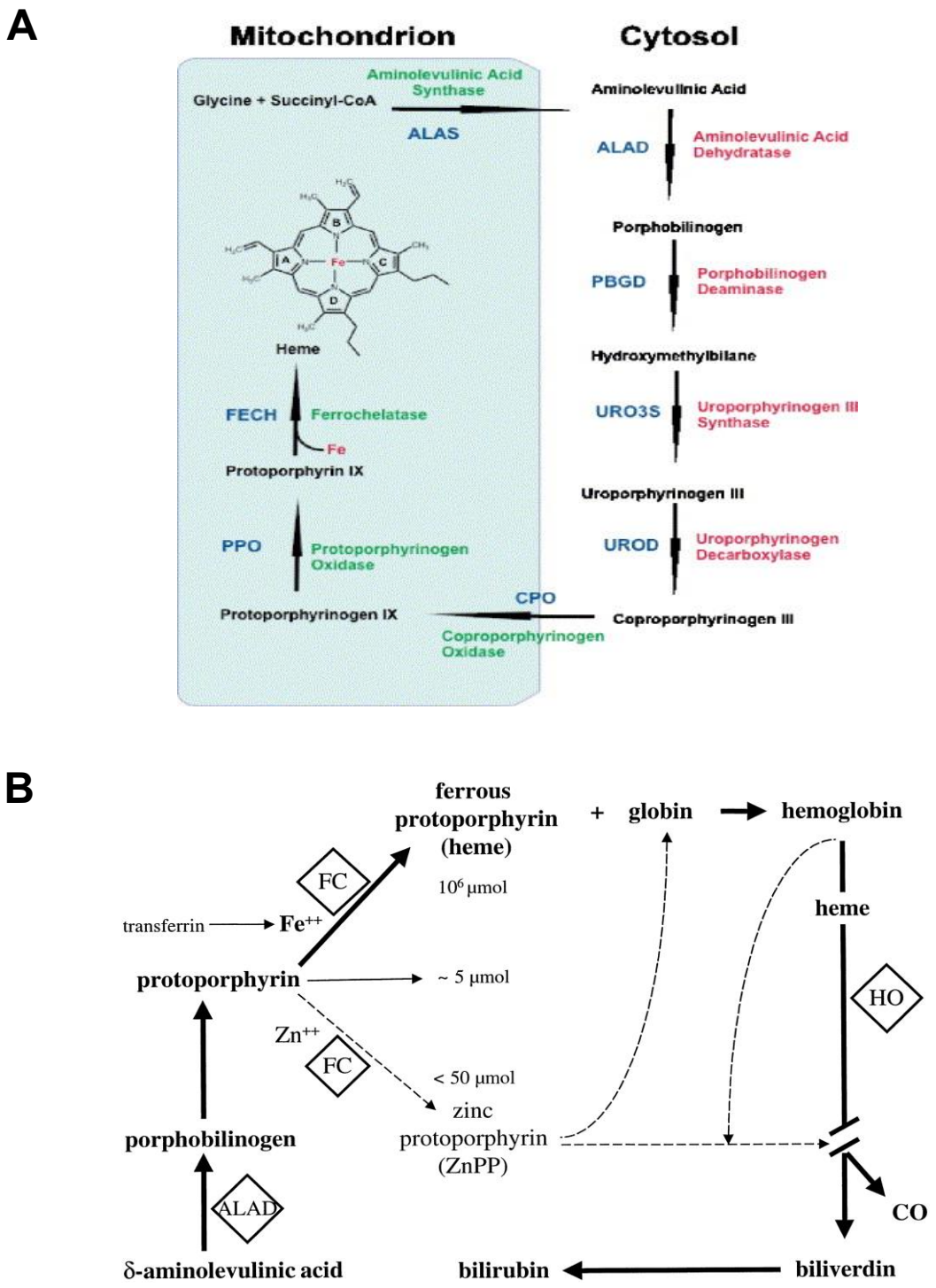


Fig. 1. Heme biosynthesis and ZnPP formation in the living body.

A. The heme biosynthetic pathway.
The source of this figure is Ajioka, Phillips, & Kushner; 2006.

B. ZnPP formation and heme metabolism. ALAD: ALA dehydratase, FC: Ferrochelatase, HO: Heme oxygenase. The source of this figure is Labbé, Vreman, & Stevenson; 1999.

suboptimum iron availability (Labbé et al., 1987; Labbé & Rettmer, 1989). The reaction to insert a zinc ion into PPIX is also catalyzed by FECH (Labbé et al., 1999). It was reported that an excess of metal-free protoporphyrin accumulated in iron deficiency, and non-enzymatically chelated zinc ions to form ZnPP (Labbé et al, 1987). However, subsequent researches on FECH revealed that this enzyme catalyzes zinc as well as iron chelation by PPIX (Taketani & Tokunaga, 1982; Bloomer et al., 1983). In addition, ZnPP may regulate heme catabolism through competitive inhibition of heme oxygenase, the rate-limiting enzyme in the heme degradation pathway that produces bilirubin and carbon monoxide. On the other hand, ZnPP concentration is also increased in association with lead toxicity (Martin et al., 2004; Hawke et al., 1992; Kasperczyk et al., 2012). Consequently, it seems that lead impairs iron utilization in an additional way i.e. lead inhibits ALA dehydrogenase thus increasing the erythrocyte concentration of PPIX (Labbé et al., 1999). Lead also inhibits the FECH, which leads to inhibition of iron incorporation in PPIX. This inhibition should result in increased free PPIX, rather than the increase in ZnPP that predominates in lead toxicity in the living body (Lamola & Yamane, 1974). However, it is doubtful whether ZnPP is formed in Parma ham by the same mechanism as in the living body.

For several years researchers have studied the mechanism of the formation of ZnPP in meat products. Although ZnPP formation mechanisms in meat products are not completely elucidated yet; three possible mechanisms have been suggested for this red pigment formation in meat products (Wakamatsu et al., 2004a; Wakamatsu et al., 2004b): a) a non-enzymatic reaction in which ZnPP is formed under anaerobic conditions; b) enzymatic reactions where FECH is directly involved; and c) bacterial enzymatic reactions. These three possibilities have been examined by many researchers.

The non-enzymatic formation of ZnPP from PPIX was clearly confirmed as a reversible reaction in acetone:water (3:1) and found to have a second-order rate constant at 35°C with an energy of activation (Becker et al., 2012). The enzymatic formation of ZnPP from myoglobin was demonstrated by the inhibitory effect of the FECH inhibitor Pb (II) and N-methylmesoporphyrin (Becker et al., 2012). It was also reported that addition of exogenous recombinant yeast FECH facilitates the production of ZnPP from myoglobin-heme and heme in meat, via the replacement of iron in the PPIX ring by zinc ions (Chau et al., 2011). It was suggested that ZnPP formation is depended on the involvement of an enzyme (Wakamatsu et al., 2007). Endogenous porcine FECH in meat is responsible for the formation of ZnPP and the formation of ZnPP is dependent upon the initial heme concentration in dry-cured ham including Parma ham (Chau et al., 2011). On the other hand, it was proposed that endogenous enzymes as well as microorganisms may be involved in ZnPP formation (Wakamatsu et al., 2004a). It was claimed that the myoglobin derivative present in matured Parma ham was formed by microbial activity in the ham during the elongated processing (Morita et al., 1996). Later, it was reported that the post-mortem accumulation of the heme biosynthesis metabolite ZnPP in porcine muscle is associated with both a meat-inherent and a bacterial enzymatic reaction during meat storage (Fig. 2) (Khozroughi et al., 2018). They presumed that ZnPP was formed by *Psudomonas fluorescens* as a part of a competitive reaction, in which some part of heme did not react with heme oxygenase (HO), but was transformed by bacterial FECH (Fe(II)-Zn(II)-substitution) to ZnPP (Fig. 3). However, because there are few microorganisms inside dry-cured ham (Toldra & Etherington, 1988), the bacterial mechanism is believed to play a minor role in the formation of ZnPP in dry-cured ham. Therefore, the mechanism by which ZnPP is formed in Parma ham might be occurred non-enzymatically or by the influence of

endogenous enzyme. Nevertheless, ZnPP formation mechanism in meat and meat products is still imprecise.

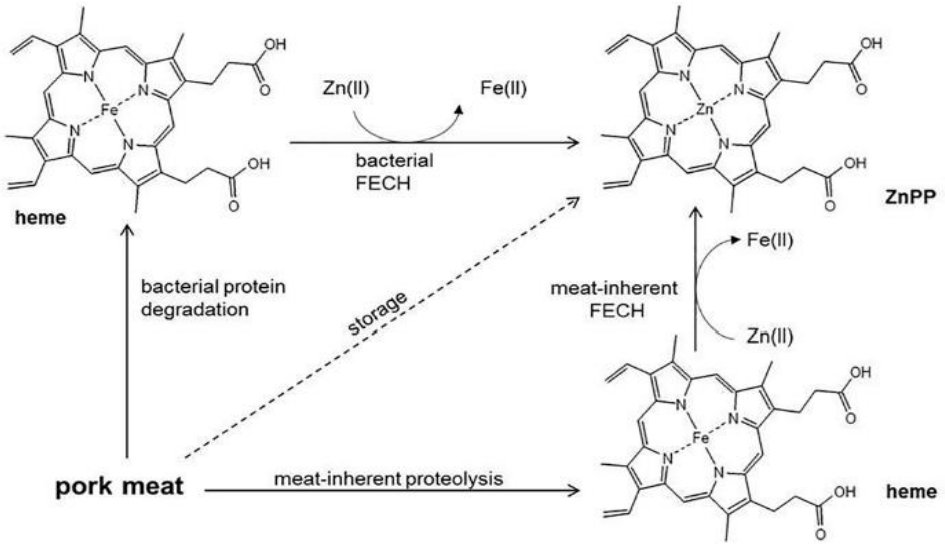


Fig. 2. Two pathways of ZnPP formation in stored pork meat by the conversion of heme from meat proteins.

Two possible pathways of FECH-induced ZnPP formation are microbiological and meat-inherent. The source of this figure is Khozroughi et al. (2018).

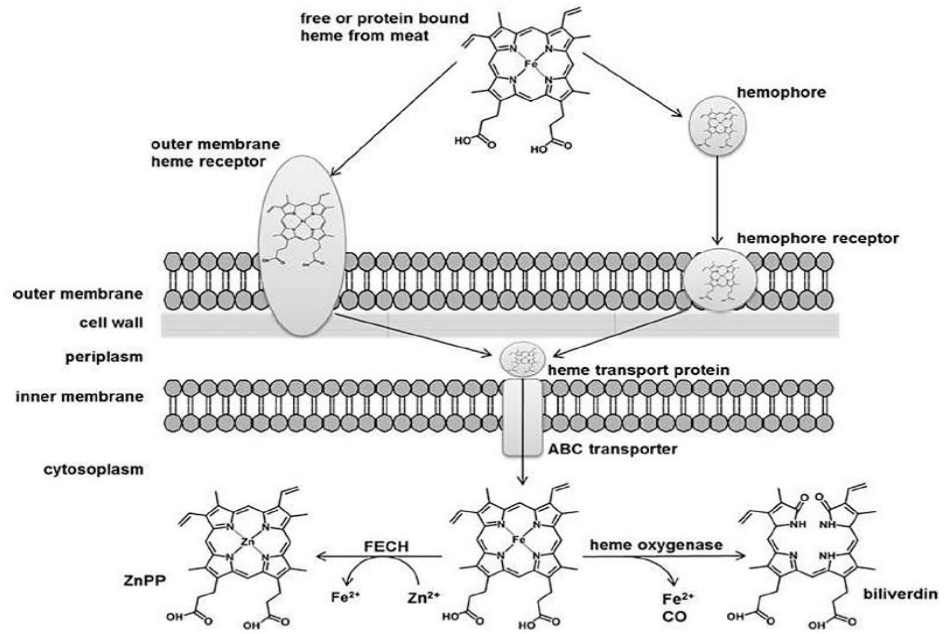


Fig. 3. Postulated reaction pathway for the ZnPP formation in *P. fluorescens* during the storage of pork meat. The source of this figure is Khozroughi et al. (2018).

In Parma ham, ZnPP was first proposed to be formed from myoglobin by a transmetallation process in which iron (II) ion is substituted by zinc (II) (Wakamatsu et al., 2004a; Wakamatsu et al., 2004b). Myoglobin is a major heme protein in meat and has been considered as a heme donor in the formation of ZnPP since the structures of ZnPP and heme are similar except iron and zinc. The heme iron of myoglobin present in post-mortem muscle tissue suggests that substitution of iron by zinc occurs in ZnPP formation (Adamsen et al., 2006b). However, Becker et al. (2012) suggested that ZnPP is formed in pork homogenates by both enzymatic and non-enzymatic reactions and the enzymatic formation of ZnPP dominated initially during the salting of the muscle, while the non-enzymatic substitution reactions mostly occur in later stages of the production processes. It was also reported that exogenous recombinant yeast FECH facilitates the production of ZnPP from myoglobin-heme and heme in meat via the replacement of iron in the protoporphyrin ring by zinc ions (Chau et al., 2011). FECH catalyzes the insertion of Fe^{2+} into PPIX in a living body but inserts other divalent metals such as zinc into porphyrins (Taketani et al., 2007; Chau et al., 2010). FECH was observed in mitochondria of Parma ham, and its localization almost coincided with that of ZnPP (Shimoda, 2014). However, because the molecular weight of FECH (42 kDa) is considerably greater than that of myoglobin (17 kDa), it is suspected that it would be difficult for FECH to assist the de-ironing of heme located in the globin pocket of myoglobin. Therefore, partial degradation of myoglobin is important for the enzymatic formation of ZnPP. However, it has been shown that ZnPP is formed by a Fe^{2+}–Zn^{2+} substitution in myoglobin heme, and that the accompanying myoglobin degradation is not obligatory (Khozroughi et al., 2017). Subsequently, Honma (2014) reported that significant myoglobin degradation was observed at pH 4.75 but the degradation of

myoglobin did not occur during ZnPP formation in model solution. Therefore, more detail examination about the role of myoglobin is required.

It was also reported that the amount of ZnPP peaked at pH 5.5 using *longissimus* muscle and decreased considerably at lower or higher pH (Wakamatsu et al., 2007). Recently, it has found a new optimum pH of ZnPP formation at 4.75 using *infraspinatus* muscle (Honma, 2014; Wakamatsu et al., 2019). The pH considerably influences enzymatic activity in biological processes. The pH of fresh meat is considered a critical factor for FECH activity in ZnPP formation. The optimum pH of FECH for iron insertion was first reported as 7.8-9.0 (Porra & Jones, 1963). Recently, the optimum pH of FECH activity ranges from 5.5-6.0 for iron removal and from 7.5-8.0 for zinc insertion as investigated using an *in vitro* meat model with porcine mitochondria (Chau et al., 2010), whereas the zinc insertion pH in raw meats is 5.5-6.0 (Adamsen et al., 2003; Adamsen et al., 2004). In addition, the optimal pH for ZnPP formation varies for different internal organs—for instance, the values are 5.0–5.5, 4.5, and 5.5–6.0 for porcine heart, liver, and kidney, respectively (Wakamatsu et al., 2015). Ishikawa et al. (2006) also reported that the enzyme activity of a porcine heart extract exhibits an optimum pH of 5.5. Accordingly, ZnPP was only formed at a pH higher than 4.9 after an extensive drying period of up to 177 days (De Maere et al., 2016), indicating that both pH and production time are critical factors for its formation in nitrate-free, dry fermented sausages. Therefore, it is unclear how FECH would facilitate ZnPP formation at the two optimum pH 5.5 and 4.75, as it requires higher pH for its Zn insertion activity.

Skeletal muscle is composed of different muscle fiber types that arise from the coordinated expression of distinct sets of structural proteins and metabolic enzymes

(Schiaffino & Reggiani, 1996; Chang et al., 2003). Vertebrate skeletal muscle contains three muscle fiber types and their relative proportions determine the muscle's overall profile (Gleeson et al., 1980). Slow-twitch (type I) muscle fibers have more mitochondria and myoglobin than fast-twitch (type IIa and IIb) fibers (Pearson & Young, 1989). The proportion of each muscle fiber type varies according to muscle function and can be characterized histologically or biochemically. However, it was also reported that meat color, which depends on muscle fiber composition, was shown to be significantly correlated with Zn chelatase activity in pork muscle (Parolari et al., 2009). Moreover, in our laboratory, it was found that the ratio of muscle fiber types, ZnPP formation and muscle pH are correlated in pork and ZnPP formation is classified into three distinct groups with optimum pH of 4.75 (Fig. 4A), 5.5 (Fig. 4B), or both (Fig. 4C) depending on the muscles (Honma, 2014). ZnPP formation was much higher at pH 4.75 in *infraspinatus* muscle compared with at pH 5.5 in *longissimus* muscle (Fig. 5). Furthermore, the amount of ZnPP formed at pH 4.75 was positively related to the ratio of type I muscle fibers in skeletal muscle (Honma, 2014). The fluorescence of ZnPP in slow-twitch (type I) muscle fibers was stronger than that in fast-twitch (type II) muscle fibers (Shimoda, 2014). Many granular ZnPP spots with strong fluorescence were observed in slow-twitch muscle fibers and coincided with the localization of mitochondria. It is therefore possible that ZnPP formation at pH 4.75 is influenced by FECH levels in mitochondria since FECH located in the inner mitochondrial membrane of skeletal muscle (Taketani, 1993).

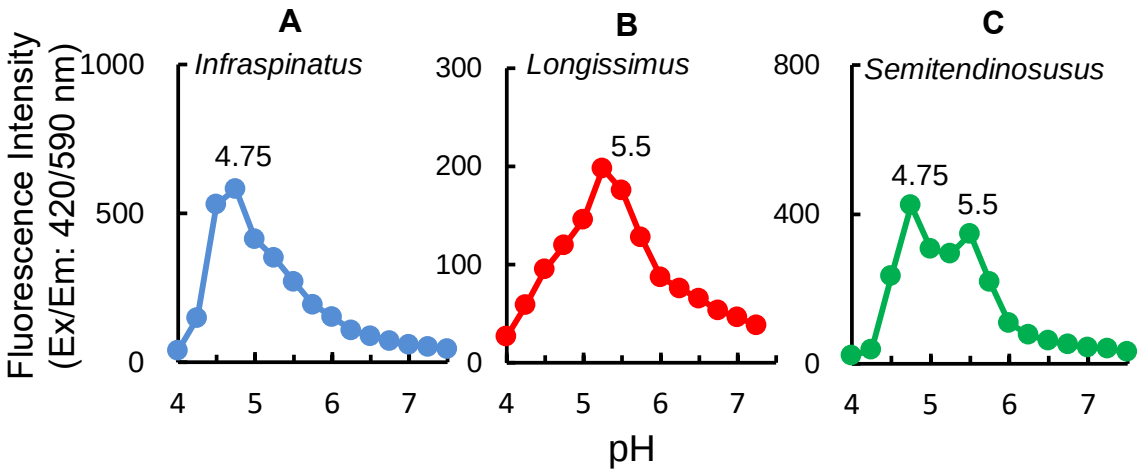


Fig. 4. Typical three patterns of optimum pH for ZnPP formation among porcine skeletal muscles (Honma, 2014).

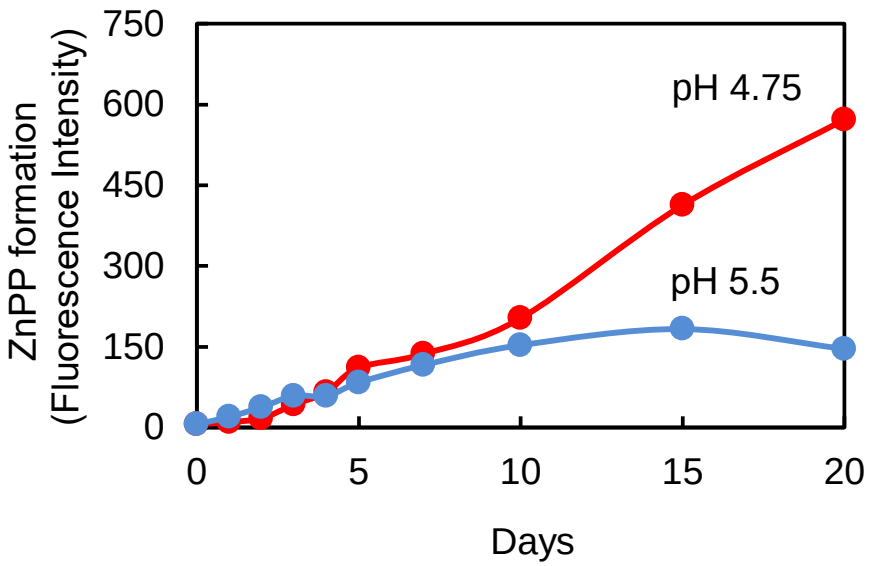


Fig. 5. ZnPP formation in optimum pH at 4.75 and 5.5 in different incubated days (Honma, 2014).

PPIX is an important precursor to biologically essential prosthetic groups such as heme. When the amounts of bioavailable iron in the reticulocyte are low, FECH inserts zinc into PPIX instead of iron, resulting in raised ZnPP (Lamola & Yamane, 1974). Accordingly, the presence of PPIX is important for the formation of ZnPP. It was reported that copropophyrinogenase catalyzes the conversion of copropophyrinogen III to protoporphyrinogen IX, subsequently protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen IX to form PPIX in the heme biosynthetic pathway (Poulson & Polglase, 1974). However, these intermediate products of heme biosynthesis are unstable and their presence in the post-mortem muscle and role in the formation of ZnPP and PPIX in meat products has not been confirmed yet. Investigations of PPIX formation using the *longissimus* muscle as an experimental system have also shown that the formation patterns of ZnPP and PPIX are similar (Wakamatsu et al., 2007). Oxygen inhibited ZnPP and PPIX formation, which was shown to peak at pH 5.5 and was suppressed by $NaNO_2$ and FECH inhibitor (Wakamatsu et al., 2007). It was also reported that ZnPP in a model solution was formed by the insertion of Zn into PPIX, which was formed independently (Wakamatsu et al., 2007). However, the effects of those factors on PPIX formation and the precursor for ZnPP and PPIX formation were not checked at pH 4.75 yet. The accumulation of PPIX during the production of meat products has only observed by De Maere et al. (2016). Possible reasons might be the absence of free zinc ions (Ishikawa et al., 2007) or the presence of chelating constituents (Benedini et al., 2008), which are able to inhibit ZnPP formation. Therefore, a full understanding of the formation of PPIX or its precursor will be worthwhile to elucidate the ZnPP formation mechanism in meat products.

Although it is assumed that FECH, myoglobin as heme donor, and Zn^{2+} play important roles in ZnPP formation in meat as above-mentioned, the precise mechanism is not well understood. FECH is located at the inner membrane of the mitochondria in mammalian cells (Taketani, 1993) and hence is relatively insoluble. On the other hand, myoglobin is distributed throughout the cytoplasm (Ordway, 2004) and is a water-soluble component. Moreover, Adamsen et al. (2006a) reported that the amount of ZnPP formed is proportional to the Zn content in dry-cured meat products. It was also reported that concentrations of Zn^{2+} up to 100 μM increased the rate of ZnPP formation in extracts of porcine heart mitochondria (Ishikawa et al., 2007). Addition of Zn^{2+} (50 μM) to mouse liver mitochondria extracts incubated for 1 h at 45°C was also found necessary in order for the formation of ZnPP (Taketani et al., 2007). A large amount of zinc exists in meat and zinc content in beef and pork is 4.6 and 2.30 mg/100 g, respectively (Moss et al., 1983). Zinc in meat is believed to exist as the free ion or as complexes with various zinc-binding proteins; the zinc source for ZnPP formation is still unclear. FECH and myoglobin are in different locations in muscle; hence, the separation of muscle fractions that contains FECH and myoglobin is important to clarify their contribution to the formation of ZnPP in meat.

To the best of our knowledge, there are no reports about the contributors to ZnPP and PPIX formation after separation of pork homogenate. In our laboratory, first Ikeda (2005) found that water-soluble and insoluble components in separated pork homogenate are required to the formation of ZnPP. Later, Shiraishi (2010) reported that insoluble fraction, high molecular water-soluble fraction and low molecular water-soluble fraction of pork *longissimus* muscle homogenate are essential for the formation of ZnPP at pH 5.5. It was suggested that myoglobin reagent did not facilitate the

formation of ZnPP and the other high molecular weight water-soluble protein(s) except myoglobin contributed to ZnPP formation (Shiraishi, 2010). Hence, plural components contribute to the formation of ZnPP at pH 5.5. It was also suggested that low-molecular components other than zinc have suggested contributing to PPIX and ZnPP formation since it was promoted PPIX formation (Shiraishi, 2010). However, the further investigations about the contributors for PPIX formation in pork homogenate and the involvement of myoglobin or other water-soluble components have not been checked yet. In addition, the previous study could not identify the contributed protein for ZnPP formation at pH 5.5 in *longissimus* muscle. On the other hand, there is no information about the contributors in the newly discovered optimum pH of ZnPP formation at pH 4.75 (Honma, 2014). The ability to form ZnPP at pH 4.75 was much higher than that at pH 5.5. There is a possibility of plural precursors or contributors involved in ZnPP formation. The precursors or the contributors might also be different at both optimum pH conditions of ZnPP formation. Therefore, separation of pork homogenate will be helpful to find out the contributors for ZnPP formation in both pH.

In the present study, it was hypothesized that ZnPP formation depends on the formation of PPIX at pH 4.75 like at pH 5.5, multiple components in pork homogenate are essential to the formation of ZnPP and PPIX at pH 4.75 and 5.5, and the contributors or the precursors for the formation of ZnPP are different between both pH conditions. Therefore, to elucidate the ZnPP formation mechanism, the objectives of this research were (a) to investigate the factors that affect ZnPP and PPIX formation at the newly discovered optimum pH 4.75, (b) to search for the contributors present in pork responsible for the formation of ZnPP and PPIX at pH 4.75 and 5.5, (c) to verify the involvement of myoglobin for the formation of ZnPP and PPIX at pH 4.75 and 5.5, and

(d) to identify the water-soluble protein(s) contributed ZnPP and PPIX formation at pH 4.75 and 5.5. For these purposes, this study examined the effects of various factors (temperature, incubation time, oxygen, $NaNO_2$, FECH inhibitor) on ZnPP and PPIX formation at pH 4.75 and determined the quantitative value of ZnPP, PPIX and heme by using ZnPP/PPIX formation model experiments. Then, the pork homogenate was fractionized and checked the ZnPP and PPIX-forming ability of the separated fractions. Furthermore, the water-soluble fraction of pork homogenate was separated by the various separation methods in order to screen the contributor(s) and precursor(s) in ZnPP and PPIX-forming ability of the separated water-soluble protein(s) at both pH 4.75 and 5.5.

2. Materials and methods

2.1. Materials

2.1.1. Sampling

Porcine *Longissimus dorsi* (LD) and *Infraspinatus* (IS) muscle were collected from three primal cuts of common pigs produced in Hokkaido. After removing fat and connective tissue, the muscle was minced, packaged and stored as frozen (-20℃) until use.

2.1.2. Antibiotic solutions

Penicillin G potassium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and streptomycin sulfate (Wako Pure Chemical Industries, Ltd.) were dissolved in ultrapure water so as to be 7 mg/ml and 25 mg/ml, then filtered through a sterile syringe filter (Minisart® syringe filter, 0.45 μm, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored frozen in aliquots (-20℃). Gentamicin sulfate (Wako Pure Chemical Industries, Ltd.) was also dissolved in ultrapure water so as to be 10 mg/ml, after the sterile filtration through a syringe filter, stored frozen in aliquots (-20℃).

2.1.3. ZnPP, PPIX, and heme standard solutions

A stock ZnPP standard solution was prepared by dissolving 10 mg of ZnPP (Aldrich Chem. Co., Milwaukee, WI, USA) in 50 ml of N, N-dimethylformamide (DMF) (Wako Pure Chemical Industries, Ltd.). After dissolving 10 mg of PPIX disodium salt (Aldrich Chem. Co.) with a few drops of 1 M HCl, a stock PPIX standard solution was prepared by dilution with 50 ml of DMF. After dissolving 10 mg of hemin (Sigma–Aldrich Co., St Louis, MO, USA) with a few drops of 5% ammonia water, a stock heme standard

solution was prepared by dilution with 50 ml of DMF. Working solutions of ZnPP, PPIX and hemin standard were diluted with methanol/ammonium acetate (pH 5.16) (86:14, v/v).

2.1.4. Oxymyoglobin and the impurities

Myoglobin (horse muscle-derived, Nacalai Tesque Inc., Kyoto, Japan) and sodium ascorbate (Kanto Chemical, Co. Inc.) were dissolved in pure water to 0.1 and 0.4 mg/ml respectively for the preparation of oxymyoglobin solution. Oxymyoglobin was dialyzed overnight against ultrapure water to remove the low molecular weight impurities to obtain desalted oxymyoglobin. Oxymyoglobin solution was subjected to ultrafiltration spin column with a 10 kDa molecular weight cut off (MWCO) membrane (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd., Stonehouse, UK) to obtain low molecular weight components. The filtrate was as the impurities of myoglobin reagent.

2.1.5. Other reagents

The chemicals and reagents used in this study are acetone (HPLC grade, Wako Pure Chemical Industries, Ltd.), ethylenediaminetetraacetic acid (EDTA) (Kanto Chemical Co., Tokyo, Japan), N-methyl mesoporphyrin IX (N-MMP) (Frontier scientific, Logan, USA), trisodium citrate dihydrate (Wako Pure Chemical Industries, Ltd.), citric acid monohydrate (Wako Pure Chemical Industries, Ltd.), sodium dodecyl sulphate (SDS) (Nacalai Tesque, Inc.), CBB R-250 (Wako Pure Chemical Corporation), acrylamide (Nacalai Tesque, Inc.), N, N, N', N'- tetramethylethylenediamine (TEMED) (Nacalai Tesque, Inc.), and ammonium peroxodisulfate (APS) (Nacalai Tesque, Inc.).

2.2. Methods

2.2.1. ZnPP and PPIX formation model system using pork homogenate

Pork IS homogenate (10%) was prepared as previously described (Wakamatsu et al., 2004a, 2007), with minor modification. About 10 g of pork IS was homogenized with 90 ml of ultrapure water by using a homogenizer (CELL MASTER CM-100, ASONE, Tokyo, Japan) at 10,000 rpm for 90 seconds. After adjusting the pH to 4.75 with diluted hydrochloric acid (HCl), antibiotics were added to final concentrations of 70 μg/ml penicillin G potassium, 250 μg/ml streptomycin sulfate, and 50 μg/ml gentamicin sulfate. PPIX formation in the homogenate was investigated after adding EDTA (final concentration of 0.5 mM) incubated anaerobically at 25°C for 5 and 10 days in darkness. An anaerobic condition was obtained by using an oxygen absorber (A-500HS, I.S.O. Inc., Yokohama, Japan) and checked with an oxygen indicator tablet. $NaNO_2$ (final concentrations of 0, 5, 10, 15, 20, 25, and 30 μM), N-MMP (final concentrations of 0, 0.05, 0.1, 0.5, 1, and 5 μM) and myoglobin were added to the homogenates before incubation. Oxymyoglobin, desalted oxymyoglobin, and the impurities were added in the model solution to the final concentration of 0.01, 0.02, and 0.05%.

2.2.2. Fractionation of IS and LD muscle

Fractionation of pork homogenate was performed as shown in Fig. 6. Pork IS and LD (20%) were separately homogenized with ultrapure water, as the above-mentioned and adjusted to pH 4.75 and 5.5 respectively with 1.2 M HCl. Then the homogenate was centrifuged (39,920 × g, himac CR20F, Hitachi Koki, Tokyo, Japan) for 20 minutes at 4°C. The supernatant was filtered through a filter paper (No. 2, 90 mm, Toyo Roshi Ltd., Tokyo, Japan) to yield the filtrate as “the soluble fraction”. The precipitate was

diluted with ultrapure water up to the initial volume and then homogenized and centrifuged in the same manner as before for two more times to completely remove the water-soluble compounds. The precipitate was homogenized with ultrapure water up to the initial volume to yield “the insoluble fraction”. Moreover, the soluble fraction was dispensed into an ultrafiltration spin column (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.) through a sterile syringe filter (Minisart® syringe filter, 0.45 μm, Sartorius Stedim Biotech GmbH). It was then separated by centrifugation (8,000 × g, 90 minutes, 4°C, himac CR20F, Hitachi Koki) and the filtrate was “the <10 kDa soluble fraction” and the residue was diluted with ultrapure water up to the initial volume and regarded “the >10 kDa soluble fraction”. After separation, the >10 and <10 kDa soluble fractions were heat-treated separately using a water bath for 30 minutes at 100°C.

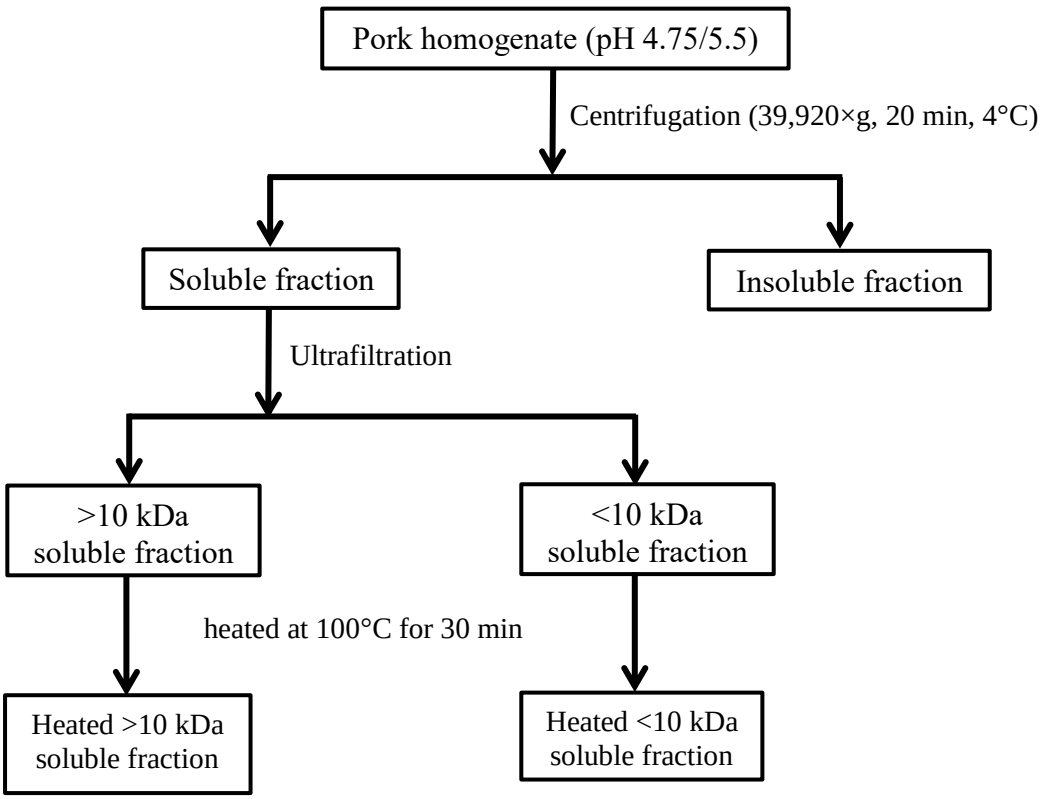


Fig. 6. Fractionation of pork homogenate.

2.2.3. Separation of water-soluble fraction by ultrafiltration

The water-soluble fraction of IS and LD muscle was subjected to ultrafiltration separately through the various ultrafiltration spin columns (10, 30, 50 and 100 kDa MWCO, VIVASPIN 6, VS06S1, Sartorius Stedim Lab Ltd). The filtrate was used as the <10, <30, <50 and <100 kDa soluble fractions respectively.

2.2.4. Separation of water-soluble protein by gel filtration chromatography

The water-soluble fraction (40 ml) of IS and LD were concentrated separately using an ultrafiltration spin column (VIVASPIN 20; 10,000 MWCO, VS1501, Sartorius Stedim Lab Ltd.) by centrifugation (8,000 × g, 90 minutes, 4°C, himac CR20F, Hitachi Koki). The concentrated >10 kDa soluble fraction (about 1 ml) was applied to a gel filtration column. The gel filtration column for IS at pH 4.75 was used Toyopearl HW 55F (3.3 φ × 60 cm, Tosoh Corporation, Tokyo, Japan) and for LD at pH 5.5 was used Toyopearl HW 50 (2.5 φ × 50 cm, Tosoh Corporation). The mobile phase consisted of 50 mM sodium chloride and 10 mM citrate buffer (pH 4.75 for IS and 5.5 for LD). The flow rate was 15 ml/h. Eluted fractions were collected in test tubes with the fraction collector, every 20 minutes (5 ml/test tube). The absorbance of the collected fractions was measured at 280 nm for protein and at 400 nm for myoglobin content by using a spectrophotometer (UV-1800, Shimadzu, Co., Kyoto, Japan).

2.2.5. Fractionation of the water-soluble fraction using ammonium sulfate

Protein from the water-soluble fraction of LD was fractionated according to “Data for Biochemical Research” by Dawson et al. (1969). The water-soluble fraction was saturated with NH_4SO_4 up to 25% saturation at 0℃. Then the sample was centrifuged at 18,000 rpm (39,920 × g) for 20 minutes at 4℃. At 25% saturation, no precipitate was

found. The 25% saturated solution was further saturated up to 40% and centrifuged to separate into supernatant and precipitate as same. A few ml of supernatant was kept as a sample. The remaining supernatant was further saturated up to 50% saturation with NH_4SO_4 and centrifuged. The same procedure was done 50-75% and 75-100% saturation with the supernatant. After each separation, the precipitate was diluted up to the initial volume. Each fraction was placed in a dialysis tube (14,000 MWCO, Viskase Companies, Inc., Tokyo, Japan) and dialyzed overnight against pure water to remove NH_4SO_4.

2.2.6. Separation of water-soluble proteins by cation exchange chromatography

2.2.6.1. Separation of water-soluble proteins by ionic strength

Pork IS and LD (20%) homogenate was adjusted to pH 4.75 (IS) and 5.5 (LD) respectively and centrifuged to separate into the soluble and the insoluble fractions as the above-mentioned. The soluble fraction (10 ml) was subjected to a cation exchange gel column (CM-Toyopearl 650M; 2 φ × 23.6 cm) equilibrated with 10 mM citrate buffer (pH 4.75 (IS)/5.5 (LD)). Then the sample was first eluted with 10 mM citrate buffer, subsequently stepwise eluted with citrate buffer including 0.15 M, 0.3 M, 0.45 M and 0.6 M NaCl. The flow rate was 1.0 ml/min. The collected fractions were stuffed into a dialysis tube (14,000 MWCO, Viskase Companies, Inc.) and concentrated by using sucrose and then dialyzed with 10 mM citrate buffer (pH 4.75/5.5) overnight separately to remove salt at 4°C.

2.2.6.2. Separation of water-soluble proteins with different pH buffer

The soluble fraction (IS and LD) was applied to a CM-Toyopearl gel column equilibrated with 10 mM citrate buffer (pH for IS 5.5-8.0 and pH for LD 4.75-8.0). Then

the column was washed with same citrate buffer separately. The unadsorbed fractions were collected. Then, the fractions were concentrated and dialyzed as the above-mentioned. The pH of the sample was adjusted to 4.75 for IS and 5.5 for LD by using HCl or NaOH solution.

2.2.6.3. Separation of water-soluble proteins with high pH buffer

The water-soluble fraction (LD) was subjected to a CM-Toyopearl gel column equilibrated with 10 mM citrate buffer of pH 8.0 (0 M NaCl). The flow rate was 1.0 ml/min. The unadsorbed fraction was collected and concentrated using aquacide II (500,000 MW, Calbiochem-Novabiochem Corporation) and adjusted to pH 7.5 with 1.2 M HCl. Then the unadsorbed fraction was again applied to the gel column equilibrated with 10 mM citrate buffer of pH 7.5 (0 M NaCl) and subsequently eluted with 10 mM citrate buffer pH 7.5 (0.6 M NaCl). The obtained fractions were concentrated separately. After concentration, the pH 7.5 buffer (0.6 M NaCl) eluted fraction was dialyzed against ultrapure water to remove the salt.

2.2.7. Model experiment for verification of ZnPP and PPIX formation by separated fractions

Regarding IS and LD muscle homogenate fractions, single fraction and mixed fractions were applied to ZnPP and PPIX formation model experiment systems as shown in Table 1. Then, antibiotics were added to the mixture of fractions and the final volume of each sample was diluted up to 1.5 ml with ultrapure water. EDTA was added to the model sample for the formation of PPIX as shown in 2.2.1. The model samples were incubated anaerobically in darkness. The incubation condition for IS was at 37°C and LD was 25°C for 5 days.

Table 1. ZnPP and PPIX formation model experiment systems

Homogenate	Insoluble fraction	Soluble fraction	>10 kDa soluble fraction	<10 kDa soluble fraction	Heated	
					>10 kDa soluble fraction	<10 kDa soluble fraction
0.5 ml	-	-	-	-	-	-
-	0.25 ml	-	-	-	-	-
-	-	0.5 ml	-	-	-	-
-	0.25 ml	0.5 ml	-	-	-	-
-	0.25 ml	-	0.5 ml	-	-	-
-	0.25 ml	-	-	0.5 ml	-	-
-	0.25 ml	-	0.5 ml	0.5 ml	-	-
-	0.25 ml	-	0.5 ml	-	-	0.5 ml
-	0.25 ml	-	-	0.5 ml	0.5 ml	-

After separation of IS and LD water-soluble fraction according to molecular weight, the separated fractions were applied to ZnPP and PPIX formation model experiment in two groups as shown in Table 2. One group was without the addition of the <10 kDa soluble fraction and another group was with the addition of the <10 kDa soluble fraction. After the addition of EDTA and antibiotics, the final volume was made up to 1.5 ml with ultrapure water. The model solutions were incubated same conditions for IS and LD as the above-mentioned.

Table 2. ZnPP and PPIX formation model experiment systems after ultrafiltration

Insoluble fraction	Separated soluble fraction (kDa)				
	<10	<30	<50	<100	
0.25 ml	0.5 ml	-	-	-	Without addition of <10 kDa
0.25 ml	-	0.5 ml	-	-	
0.25 ml	-	-	0.5 ml	-	
0.25 ml	-	-	-	0.5 ml	
0.25 ml	0.5 ml	0.5 ml	-	-	With addition of <10 kDa
0.25 ml	0.5 ml	-	0.5 ml	-	
0.25 ml	0.5 ml	-	-	0.5 ml	

Other separated fractions were applied to the model experiment as separated fraction 0.5 ml, insoluble fraction 0.25 ml, <10 kDa soluble fraction 0.5 ml and after the addition of EDTA and antibiotics, the final volume diluted up to 1.5 ml with ultrapure water. The model solutions were incubated same conditions for IS and LD muscle as the above-mentioned.

Oxymyoglobin and desalted oxymyoglobin were added instead of the >10 kDa soluble fraction to the final concentration of 0.01, 0.02 and 0.05%. The model solutions were incubated same conditions for IS and LD muscle as the above-mentioned.

2.2.8. Fluorescence analysis

Acetone extraction and fluorescence analysis were performed as previously described (Wakamatsu et al., 2004a). After the addition of cold acetone (3 volumes of

the sample), the sample was kept in the refrigerator for 30 minutes. Then, the sample was filtered through a filter paper (No. 2, 90 mm, Toyo Roshi Kaisha Ltd., Tokyo, Japan) and the fluorescence intensity of the filtrate was measured by using a spectrofluorophotometer (RF-5300PC; Shimadzu Co., Kyoto, Japan). Fluorescence intensity at 590 nm for excitation at 420 nm was taken as the amount of ZnPP formed, and fluorescence intensity at 630 nm for excitation at 410 nm was regarded as the amount of PPIX formed. All operations were carried out in darkness insofar as possible.

2.2.9. Quantitative analysis of ZnPP, PPIX, and heme

ZnPP, PPIX, and heme contents were determined by high-performance liquid chromatography (HPLC) as previously described (Wakamatsu et al., 2009a). Briefly, after extracting porphyrins with four volumes of acetic acid/ethyl acetate (1:4, v/v), the supernatant of each mixture was combined with an equal volume of a mobile phase consisting of methanol/1 M ammonium acetate (pH 5.16) (84:16, v/v). An STR ODS-II column (4.6ϕ × 150 mm, particle size 5 μm; Shinwa Chemical Industries, Kyoto, Japan) was used at a flow rate of 1.0 ml/min at 35°C. ZnPP was detected by measuring the fluorescence of excitation at 420 nm and emission at 590 nm, and PPIX was detected by excitation and emission wavelengths of 410 and 630 nm, respectively. Heme was detected by absorbance at 400 nm. All steps in the procedure were carried out in darkness insofar as possible.

Table 3. HPLC settings

Item		Configuration
Flow rate		1.0 ml/minute
Injection volume		50 µl
Column temperature		35°C
Recorder settings （UV-VIS detector）	Ratio range	10
	Thresh hold	0.0001 AU
Output (UV-VIS detector)	Strength unit	Volt
	AUX range	0.5 AU/V
	Recorder output range	0.005
Output (spectral fluorescence detector)	Gain	×4
	Sensitivity	High
	Recorder output range	1

2.2.10. SDS-PAGE

An equal volume of SDS treatment solution composed of 100 mM Tris-HCl (pH 6.8), 2% SDS, 2% β-mercaptoethanol (β-ME), 40% glycerol and 0.1% bromophenol blue were added to each eluted fraction. The mixture was heated for 3 minutes at 105°C and stored at -20°C until use. Separating gel (15%) consisted with 30% acrylamide solution (29.2% acrylamide, 0.8% N, N'-methylene bisacrylamide), 1.5 M Tris-HCl (containing 0.4% SDS, pH 8.8) and ultrapure water mixing in a ratio of 2: 1: 1. APS solution (10%) and TEMED were added to the mixture as 60 µl and 10 µl, respectively. Then, the mixture was immediately poured into an electrophoresis plate (thickness 1.0 mm, ATTO, Tokyo, Japan) and was polymerized at room temperature. Stacking gel (4.5%) consisted of 30% acrylamide solution, 0.5 M Tris-HCl (containing 0.4% SDS,

pH 6.8) and pure water mixing in a ratio of 3: 5: 12. Then 20 µl of 10% APS solution and 10 µl of TEMED were added and the mixture was immediately poured into an electrophoresis plate and the sample comb was inserted. The completely polymerized gel with plate was applied to the electrophoresis apparatus (AE-6530P, ATTO) with SDS-PAGE electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). After applying the sample electrophoresis was done and then stained overnight with the conventional CBB method. The gel was photographed with the ChemiDoc XRS Plus System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.2.11. Amino acid sequencing

Protein samples were loaded onto a 15% polyacrylamide gel. Immediately after electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) by using a semi-dry transfer system (AE-6677; ATTO Co.) according to the manufacturer's manual. The applied current was 2 mA/cm^2 for 30-60 minutes. After transferring, the membrane was stained with CBB-R250 and destained with 35% methanol and 10% acetic acid solution. After destaining, the desired band was cut and transferred to micro tube. Then, it was washed with methanol and water to remove the stain. The amino acid sequencing was performed in Global Facility Center, Creative Research Institution, Hokkaido University.

2.2.12. Statistical analysis

Data are expressed as mean ± standard error. Statistical analyses were performed using Microsoft Excel 2007 with Ekuseru-Toukei 2006 (Social Survey Research Information Co., Ltd., Tokyo, Japan) for add-in software. Differences among individuals

were evaluated by one-way analysis of variance with Tukey's multiple comparison tests. Pearson's correlation was used to determine the relationship between ZnPP and PPIX formation, and the myoglobin content. $P < 0.05$ was considered statistically significant. For gel filtration chromatography data, ZnPP and PPIX were expressed as average values of the fractions. Each experiment was conducted for three different times.

3. Results

3.1. Mechanism of ZnPP formation in IS muscle at pH 4.75

Recently, the new optimum pH 4.75 for ZnPP/PPIX formation was discovered in pork (Honma, 2014). To elucidate ZnPP/PPIX formation mechanism at pH 4.75, this study examined the various factors that affect ZnPP/PPIX formation at pH 4.75. IS muscle homogenate was fractionized to investigate the contributors to ZnPP/PPIX formation at pH 4.75. Then, I focused on the water-soluble fraction including myoglobin i.e. a main heme protein in meat and evaluated the ZnPP/PPIX forming ability of the water-soluble protein that was separated by using various chromatography techniques. Since ZnPP/PPIX formation in muscle at pH 4.75 was the most obvious among pork muscles (Honma, 2014), IS muscle was used in this section.

3.1.1. Factors affecting ZnPP formation

In order to clarify the factors that affect ZnPP formation at the optimum pH 4.75, the effect of temperature, incubation time, oxygen, and FECH inhibitors ($NaNO_2$ and N-MMP) on ZnPP formation at pH 4.75 was observed by using an established model experiment system (Wakamatsu et al., 2004a). After incubation at 4, 18, 25 and 37°C for 5 days, the amount of ZnPP formed was increased to the greatest extent at 37°C (Fig. 7A). The ZnPP formation was slightly increased during the first 3 days of incubation but rapidly increased after the third day (Fig. 7B). Oxygen significantly inhibited ZnPP formation at pH 4.75 (Fig. 7C). Moreover, the addition of >5 μM $NaNO_2$ significantly inhibited the formation of ZnPP (Fig. 7D). FECH inhibitor N-MMP (Dailey & Fleming, 1983) also significantly inhibited the formation of ZnPP at pH 4.75 (Fig. 7E). These study results are similar to ZnPP and PPIX formation at pH

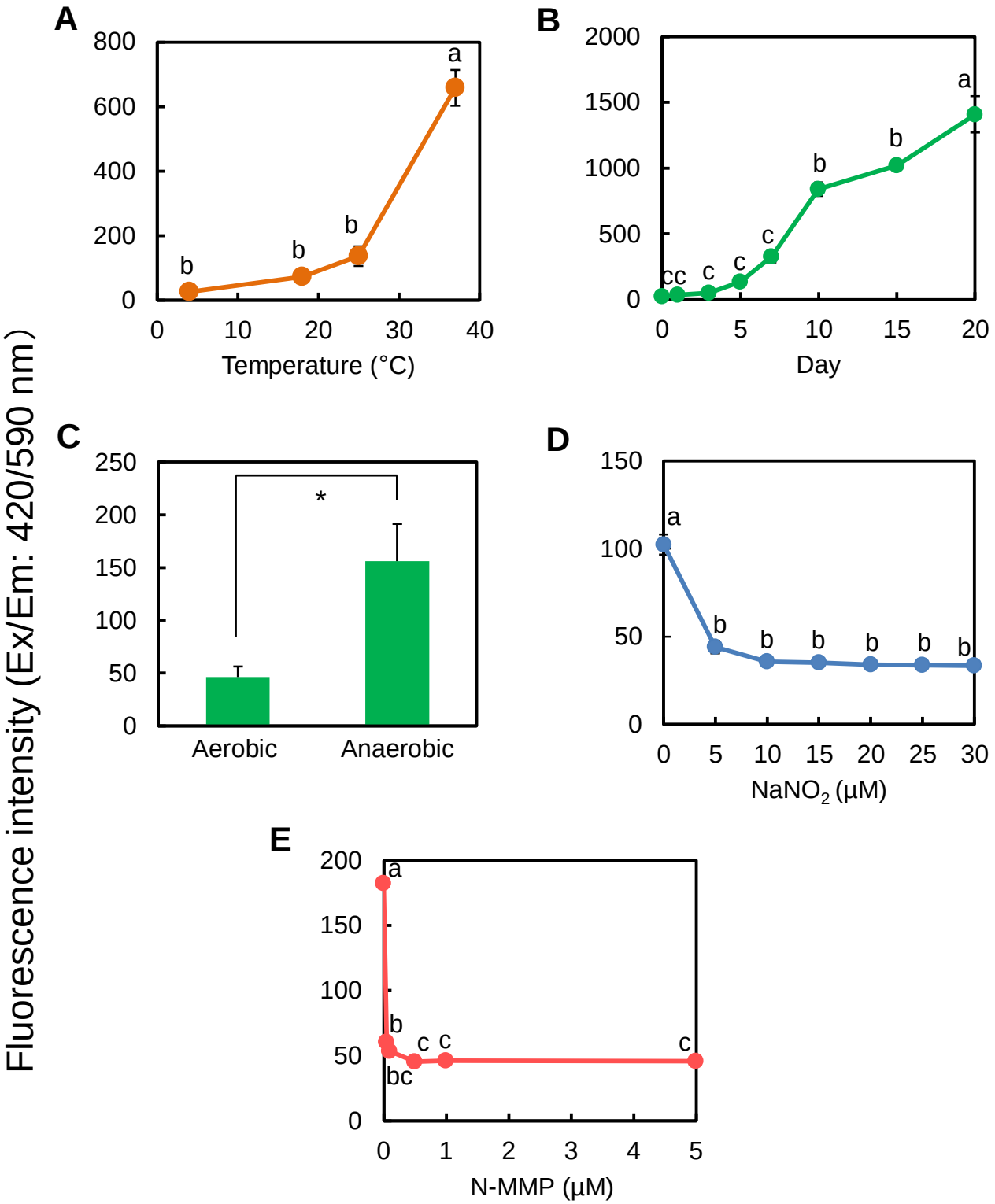


Fig. 7. Effects of temperature (A), day (B), oxygen level (C), $NaNO_2$ concentration (D), and N-MMP concentration (E) on ZnPP formation in IS muscle homogenate.

IS muscle homogenate was adjusted to pH 4.75. After the addition of antibiotics and other reagents, the samples were incubated anaerobically (except C) in the dark at 25°C for 5 days (except B). ZnPP was extracted with 75% acetone method and the fluorescence intensity (Ex/Em: 420/590 nm) was measured. Bars represent the standard error of the means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$). *Significant difference at $P < 0.05$.

5.5 (Wakamatsu et al., 2004a, 2007), except for the effect of temperature and incubation time. The optimal temperature at pH 5.5 was lower (25°C) (Wakamatsu et al., 2007) than that at pH 4.75 (37°C). Thus, ZnPP formation mechanism is strongly influenced by temperature and incubation time at a given pH suggesting that ZnPP formation is different at pH 4.75 and 5.5 in pork. These results revealed that the formation of ZnPP is affected by various factors such as temperature, incubation time, oxygen and FECH enzyme inhibitors at pH 4.75 like at pH 5.5.

3.1.2. Factors affecting PPIX formation

Grinstein & Watson (1943) reported that ZnPP is formed by enzymatic insertion of zinc into PPIX by FECH in the living body. In our earlier studies, it was also reported that ZnPP formation was strongly influenced by PPIX formation at pH 5.5 in pork (Wakamatsu et al., 2007). But, at the new optimum pH 4.75, there is no information about the PPIX formation. Therefore, this study investigated the effect of various factors on the PPIX formation in IS meat homogenate at pH 4.75 for 5 and 10 days of incubation. The PPIX formation at pH 4.75 was higher within the range of pH examined at 5 days (Fig. 8A). After the incubation for 10 days, the increase of PPIX around pH 4.75 was the most obvious. The result almost corresponded with the previous study (Honma, 2014). The amount of PPIX increased at higher incubation temperatures within the range of temperatures examined and reached a maximum at 37°C for both 5 and 10 days (Fig. 8B). The amount of PPIX formed slightly increased during the first 3 days of incubation but rapidly increased from day 3 to 20 (Fig. 8C). Oxygen inhibited the formation of PPIX up to 10 days (Fig. 8D). Furthermore, $NaNO_2$ at concentrations >5 µM almost suppressed PPIX formation at pH 4.75 both in 5 and 10 days (Fig. 8E). Although the formation of PPIX was also significantly inhibited in the presence of >0.5

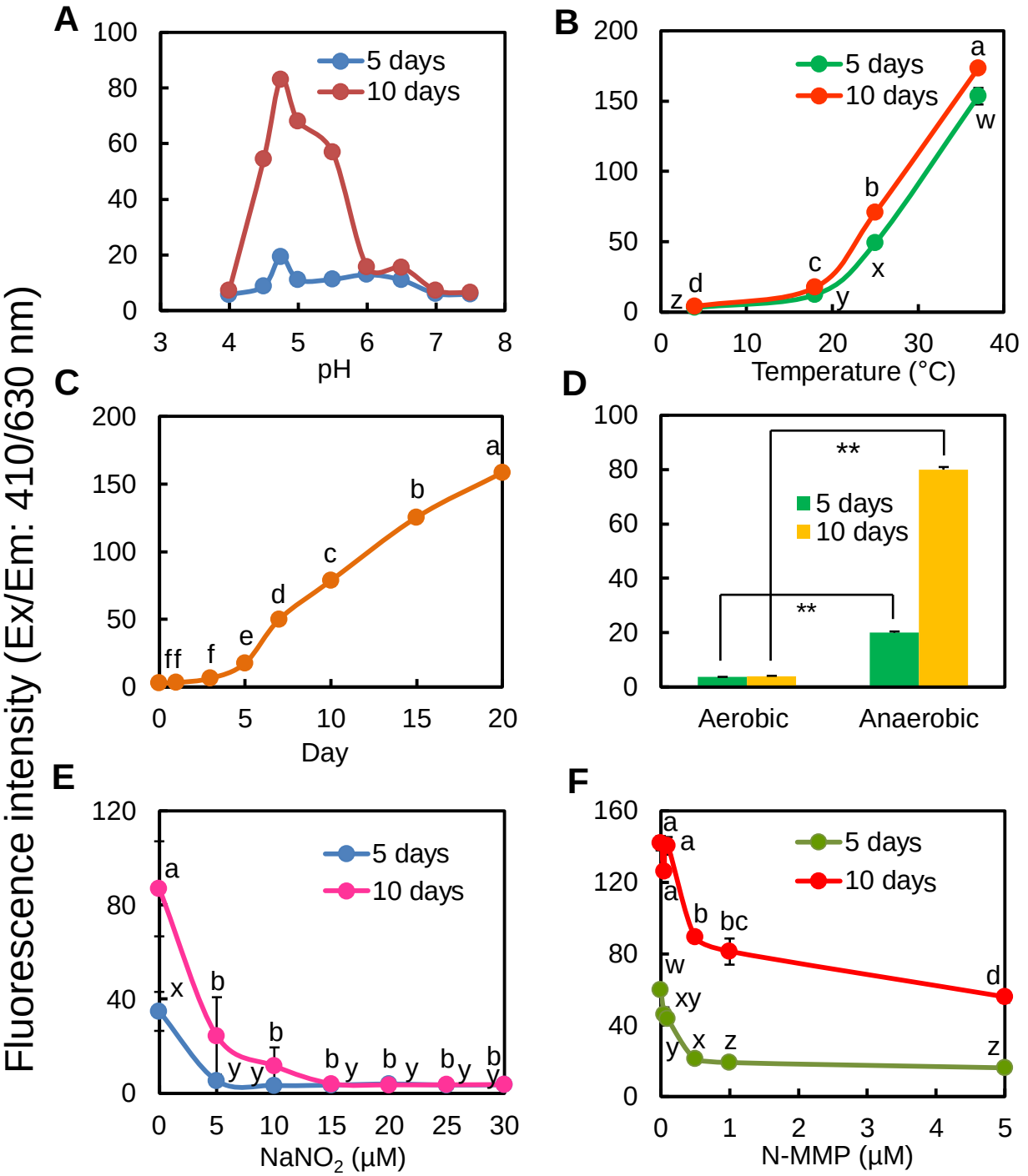


Fig. 8. Effects of pH (A), temperature (B), incubation time (C), oxygen level (D), $NaNO_2$ concentration (E), and N-MMP concentration (F) on PPIX formation in IS muscle homogenate at 5 and 10 days.

IS muscle homogenate was adjusted to pH 4.75. After the addition of antibiotics, EDTA and other reagents, the samples were incubated anaerobically (except D) in the dark at 25°C for 5 and 10 days (except C). PPIX was extracted with 75% acetone method and the fluorescence intensity (Ex/Em: 410/630 nm) was measured. Bars represent the standard error of the means (n = 3). abcdef & wxyz: Values in the same graph bearing different letters differ significantly ($P < 0.05$). **Significant difference at $P < 0.01$.

μM N-MMP, it was not completely inhibited in both 5 and 10 days (Fig. 8E). N-MMP completely inhibits the activity of FECH (Dailey & Fleming, 1983), whereas nitric oxide (NO) derived from $NaNO_2$ destroys [2Fe-2S] clusters, the active site of FECH (Sellers et al., 1996). Temperature, incubation time, oxygen, $NaNO_2$, and N-MMP affected not only ZnPP formation but also PPIX formation at pH 4.75, the aspects of ZnPP and PPIX formation were almost similar. Therefore, in the formation mechanism at pH 4.75 it was suggested PPIX was a precursor of ZnPP and PPIX formation is a key reaction in ZnPP formation.

3.1.3. Changes in heme, ZnPP, and PPIX amounts

Next, to investigate whether PPIX was formed from the demetalation of heme or independently at pH 4.75; the heme, ZnPP and PPIX contents in the model solutions with and without the addition of EDTA were determined before and after incubation (Table 4). Although the amount of ZnPP increased after incubation for 5 and 10 days in the absence of EDTA, the decrease in heme content was not observed. PPIX was not detected in the model solution before or after incubation without EDTA (data not shown). Although the presence of EDTA almost completely inhibited the formation of ZnPP and increased the amount of PPIX, there was no change in heme content corresponding to the increase in the amounts of ZnPP and PPIX formed at pH 4.75.

3.1.4. Effect of fractionation on ZnPP and PPIX formation

In order to find out the contributors to ZnPP and PPIX formation at pH 4.75, IS muscle homogenate was fractionated and the effect of separated fractions on ZnPP and PPIX formation were investigated. Since the ZnPP and PPIX formation were optimum at 37°C incubation temperature as shown in sections 3.3.1. and

Table 4. Heme, ZnPP and PPIX concentrations (µM) in model solutions "*infraspinatus*" with or without addition of EDTA before and after incubation at pH 4.75

Days	Without EDTA		With EDTA		
	Heme	ZnPP	Heme	ZnPP	PPIX
0	44.00 ± 0.28	0.04 ± 0.01^{c}	43.45 ± 1.32	0.01 ± 0.00^{b}	0.01 ± 0.00^{c}
5	45.00 ± 0.15	0.34 ± 0.02^{b}	44.03 ± 1.04	0.02 ± 0.00^{a}	0.35 ± 0.00^{b}
10	44.38 ± 0.46	1.22 ± 0.02^{a}	44.00 ± 0.47	0.02 ± 0.00^{ab}	0.76 ± 0.00^{a}

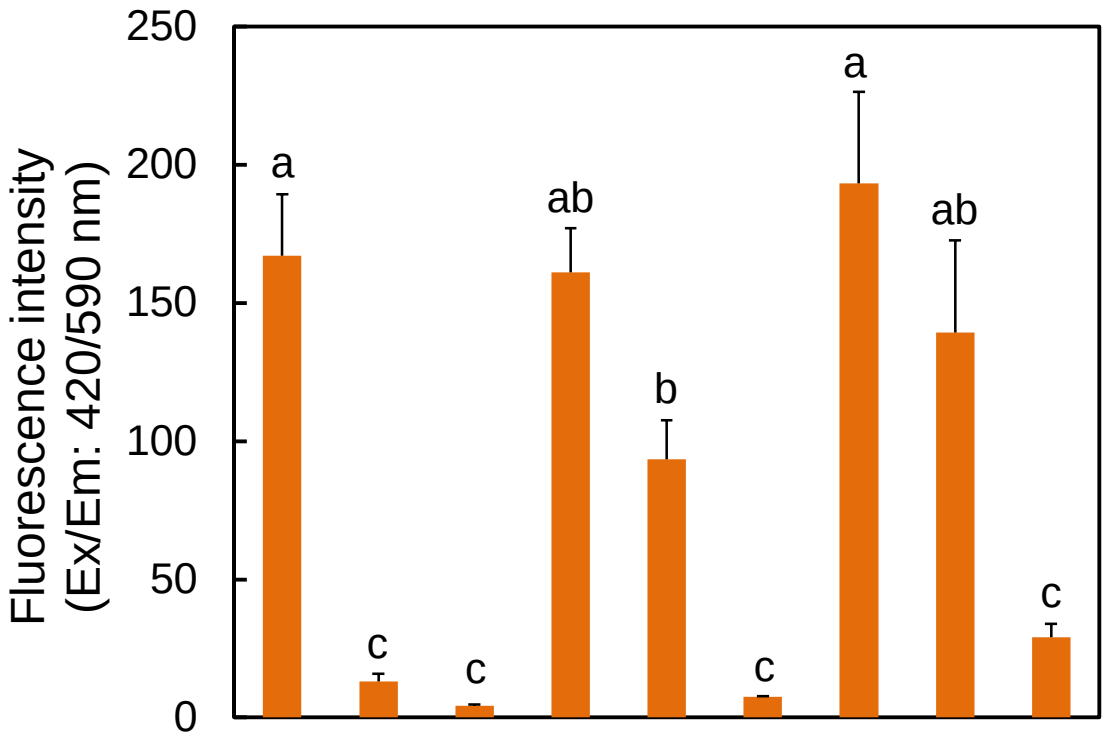
Values are means ± SEM.
^{abc}Values for each experiment bearing different letters differ significantly ($P < 0.01$).

3.3.2., the following model experiment systems were performed with incubation temperature at 37°C.

When IS muscle homogenate was fractionated into soluble and insoluble fractions and incubated separately, ZnPP formation was significantly suppressed (Fig. 9). However, when they were mixed together, ZnPP formation was rescued. When the soluble fraction was further fractionated into the >10 and <10 kDa soluble fractions and incubated with the insoluble fraction separately, ZnPP formation was significantly suppressed. However, when the separated two soluble fractions were mixed and incubated together with the insoluble fraction, ZnPP was rescued to a level similar to IS meat homogenate. Regarding PPIX formation by the fractionized IS muscle homogenate, the same behaviors were observed (Fig. 10). Therefore, this study revealed that two water-soluble fractions (>10 and <10 kDa) and insoluble fractions of IS homogenate are essential in model solution to ZnPP and PPIX formation at pH 4.75.

3.1.5. Effect of heat treatment of fractionated water-soluble fractions on ZnPP and PPIX formation

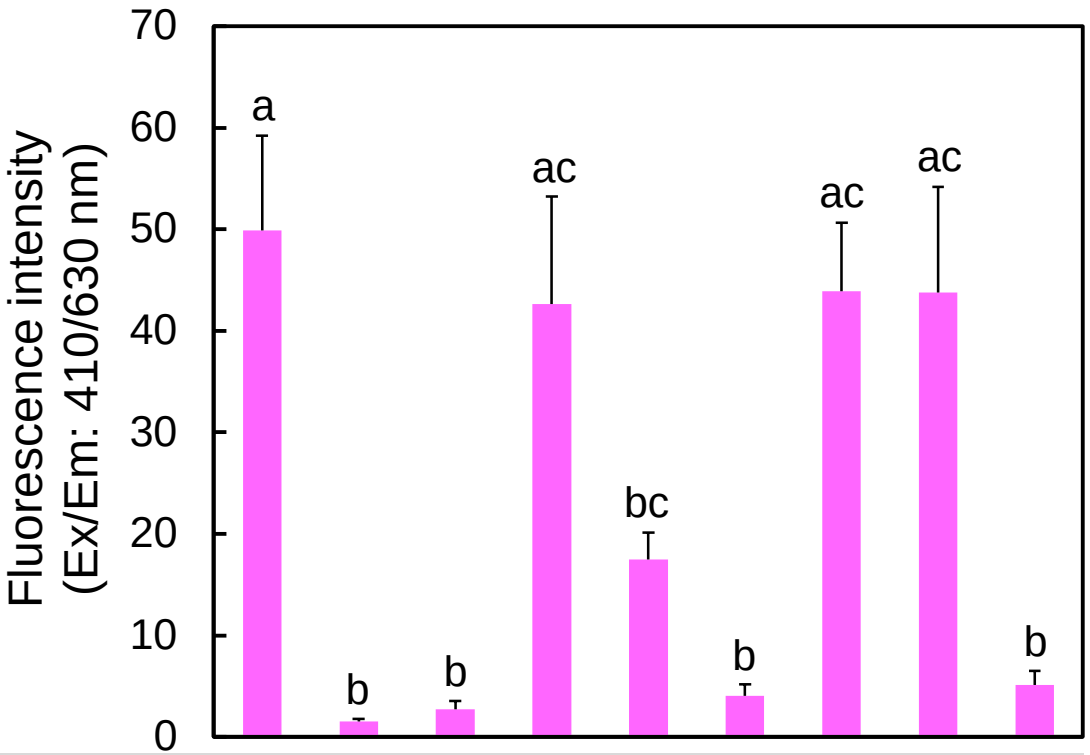
Hamm and Deatherage (1960) reported that about 77% of the water-soluble globular proteins of the bovine sarcoplasm are denatured by heating at 60°C for 30 minutes. It was also reported that the percentage of denaturation of sarcoplasmic proteins was enhanced by a heating temperature in the range of 50-80°C (Okayama et al., 1991). Since most of the proteins are assumed to present in the >10 kDa soluble fraction of pork homogenate, the heating effect of the >10 kDa soluble fraction was examined to investigate the contribution of proteins presents in the >10 kDa fraction on ZnPP (Fig. 9) and PPIX (Fig. 10) formation. When the heated >10 kDa soluble fraction was incubated with the insoluble and <10 kDa soluble fractions, ZnPP



Pork homogenate	O	-	-	-	-	-	-	-	-
Insoluble fraction	-	-	O	O	O	O	O	O	O
Soluble fraction	-	O	-	O	-	-	-	-	-
>10 kDa	-	-	-	-	O	-	O	O	-
<10 kDa	-	-	-	-	-	O	O	-	O
Heated >10 kDa	-	-	-	-	-	-	-	-	O
Heated <10 kDa	-	-	-	-	-	-	-	O	-

Fig. 9. Effect of fractionation on ZnPP formation in IS muscle at pH 4.75.

IS muscle homogenate (pH 4.75) was separated into the water-soluble and insoluble fractions. The water-soluble fraction was separated into the >10 and <10 kDa soluble fractions and heated separately. After the addition of antibiotics to single and mixed fractions, it was incubated anaerobically in dark for 5 days at 37°C. ZnPP was extracted with 75% acetone method and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$).



Pork homogenate	O	-	-	-	-	-	-	-	-
Insoluble fraction	-	-	O	O	O	O	O	O	O
Soluble fraction	-	O	-	O	-	-	-	-	-
>10 kDa	-	-	-	-	O	-	O	O	-
<10 kDa	-	-	-	-	-	O	O	-	O
Heated >10 kDa	-	-	-	-	-	-	-	-	O
Heated <10 kDa	-	-	-	-	-	-	-	O	-

Fig. 10. Effect of fractionation on PPIX formation in IS muscle at pH 4.75.

IS muscle homogenate (pH 4.75) was separated into the water-soluble and insoluble fractions. The water-soluble fraction was separated into the >10 and <10 kDa soluble fractions and heated separately. After the addition of EDTA and antibiotics to single and mixed fractions, it was incubated anaerobically in dark for 5 days at 37°C. PPIX was extracted with 75% acetone method and the fluorescence intensity was measured. Bars represent the standard error of means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

was significantly inhibited (Fig. 9). In case of PPIX formation, a similar result was obtained (Fig. 10). Therefore, this study suggested that water-soluble protein(s) present in the >10 kDa soluble fraction contribute to ZnPP as well as PPIX formation at pH 4.75.

Next, in order to clarify whether the heat-stable or heat-labile <10 kDa soluble component contributes to the formation of ZnPP and PPIX, the heating effect of the <10 kDa soluble fraction on ZnPP (Fig. 9) and PPIX (Fig. 10) formation were also examined. When the heated <10 kDa soluble fraction was incubated with the insoluble and >10 kDa soluble fractions, ZnPP formation were not suppressed to the extent seen for the corresponding experiment with the heated >10 kDa soluble fraction (Fig. 9). Heating of the <10 kDa soluble fraction has not also affected the formation of PPIX like ZnPP (Fig 10). Therefore, this study speculated that heat-stable components present in the <10 kDa soluble fraction might contribute to ZnPP as well PPIX formation at pH 4.75.

3.1.6. Effect of separation of water-soluble fraction according to molecular weight on ZnPP and PPIX formation

Next, in order to examine the contribution of the water-soluble protein(s) to ZnPP and PPIX formation, the approximate molecular weight of the water-soluble protein(s) to ZnPP and PPIX formation at pH 4.75 was investigated. The water-soluble fraction was separated by ultrafiltration and their ZnPP and PPIX-forming ability were measured (Fig. 11). After separation of the <10, <30, <50 and <100 kDa soluble fractions, the separated fractions were incubated separately with the insoluble fraction. ZnPP (Fig. 11A) and PPIX (Fig. 11B) formation were significantly lower in the <10 kDa soluble fraction group compared to the control and other soluble fractions groups

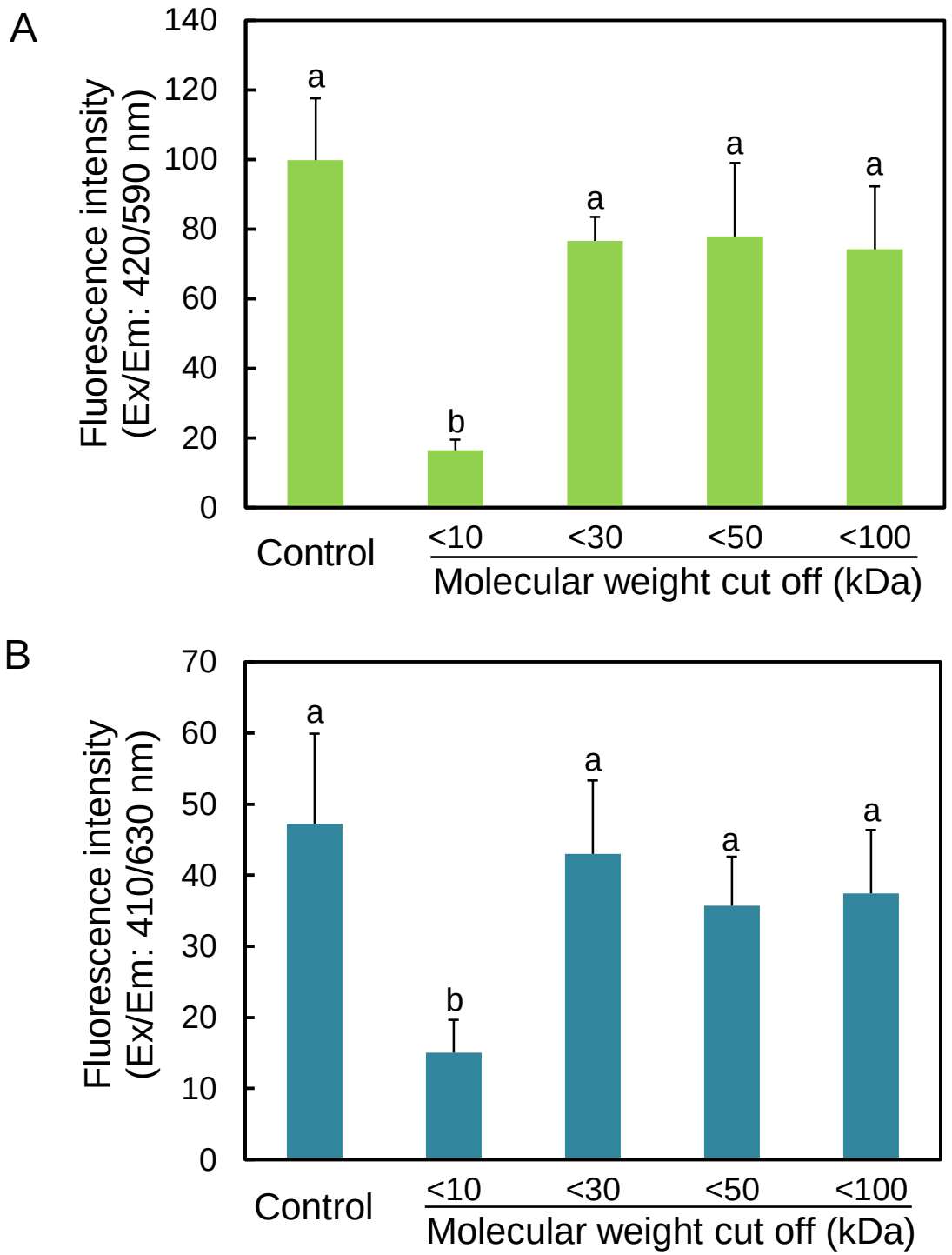


Fig. 11. Effect of ultrafiltration of water-soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated IS muscle at pH 4.75.

The water-soluble fraction of IS muscle homogenate (pH 4.75) was subjected to ultrafiltration through a molecular weight cut off 10, 30, 50 and 100 kDa ultrafiltration spin column. The filtrate was mixed with the insoluble fraction. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of means (n = 3). ab: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

(<30, <50 and <100 kDa). ZnPP and PPIX formation were increased in the <30 kDa soluble fraction group and remained same in the <50 and <100 kDa soluble fraction groups. Therefore, the present study suggested that water-soluble protein(s) having molecular weights above 10 kDa and less than 30 kDa contribute to ZnPP and PPIX formation at pH 4.75.

Further, the supplemental effect of the <10 kDa soluble fraction on ZnPP and PPIX formation in separated molecular weight groups was examined (Fig. 12). When the separated water-soluble components (the <30, <50 and <100 kDa soluble fractions) were incubated separately with the insoluble and <10 kDa soluble fractions, ZnPP (Fig. 12A) and PPIX (Fig. 12B) formation were the same as control in all groups. As the three fractionated molecular weight groups (the <30, <50 and <100 kDa soluble fractions) were contained the <10 kDa soluble fraction in common, the introduction of the additional <10 kDa soluble fraction did not lead to the formation of additional ZnPP and PPIX. In other words, the soluble <30, <50 and <100 kDa fractions contained sufficient amount of the <10 kDa soluble fractions for maximal ZnPP and PPIX formation at pH 4.75.

3.1.7. Effect of exogenous myoglobin on ZnPP and PPIX formation

As shown in section 3.1.6., the soluble 10-30 kDa fraction was essential to the formation of ZnPP and PPIX, in which myoglobin (17 kDa) exists. Additionally, Grossi et al. (2014) reported that the degradation of myoglobin was essential for the formation of ZnPP. But there has not been any direct proof about the contribution of myoglobin to ZnPP formation yet. Moreover, the contribution of myoglobin to PPIX formation was not checked yet. Therefore, in order to verify the involvement of myoglobin in ZnPP and PPIX formation at pH 4.75, the effect of

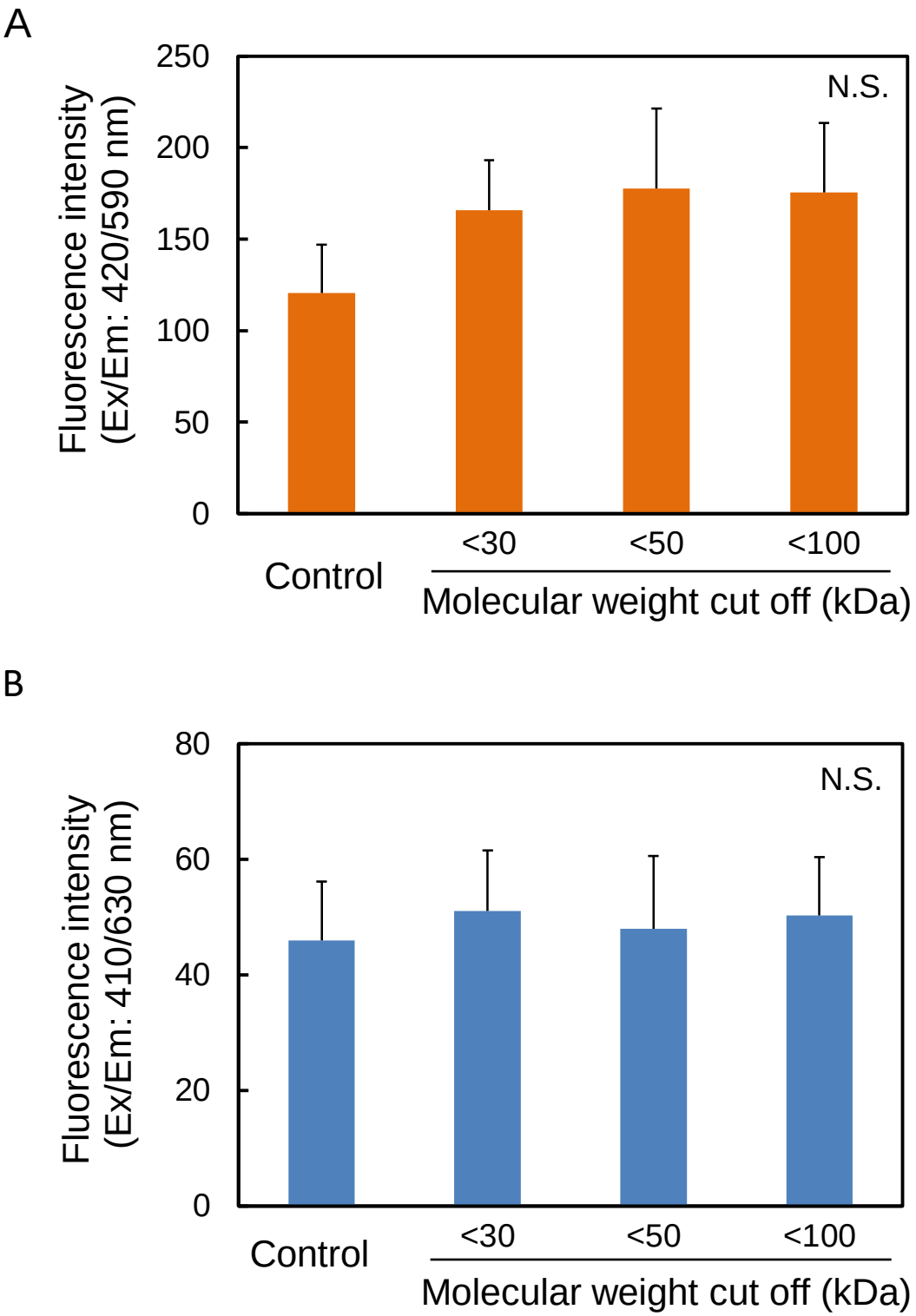


Fig. 12. Supplemental effect of <10 kDa soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated IS muscle at pH 4.75.

The water-soluble fraction was subjected to ultrafiltration through a molecular weight cut off 30, 50 and 100 kDa ultrafiltration spin column. Each filtrate was mixed with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of means (n = 3). N.S.: Non-significant.

myoglobin on ZnPP and PPIX formation studied using commercially available myoglobin reagent. Furthermore, the reduced state of myoglobin i.e. oxymyoglobin was used in this study.

3.1.7.1. Effect of exogenous myoglobin and the impurities on ZnPP and PPIX formation

First, the effect of exogenous myoglobin on ZnPP and PPIX formation in pork homogenate at pH 4.75 was investigated (Fig. 13). The addition of 0.01% oxymyoglobin facilitated the formation of ZnPP and PPIX but with the increase of oxymyoglobin content the amount of ZnPP (Fig. 13A) and PPIX (Fig. 13B) formed in pork homogenate were decreased. This result was dissimilar with the result reported by Ishikawa et al. (2006). The myoglobin reagent used in this study might contain impurities. In addition, the reducing agent, sodium ascorbate for the reduction of myoglobin might affect the formation of ZnPP and PPIX. In order to examine the effect of impurities present in oxymyoglobin solution, the impurities separated by ultrafiltration were applied to ZnPP/PPIX formation model experiment systems. The result revealed that the impurities presented in myoglobin reagent facilitated the formation of ZnPP (Fig. 14A) and PPIX (Fig. 14B), but the high concentration inhibited ZnPP and PPIX formation. However, when desalted oxymyoglobin (impurities removed) was added to IS muscle homogenate, the amount of ZnPP (Fig. 15A) and PPIX (Fig. 15B) formed were not increased linearly depending on the concentration but significantly increased than that of only IS muscle homogenate. Therefore, these results suggested that myoglobin assists the formation of ZnPP and PPIX in the IS muscle homogenate at pH 4.75.

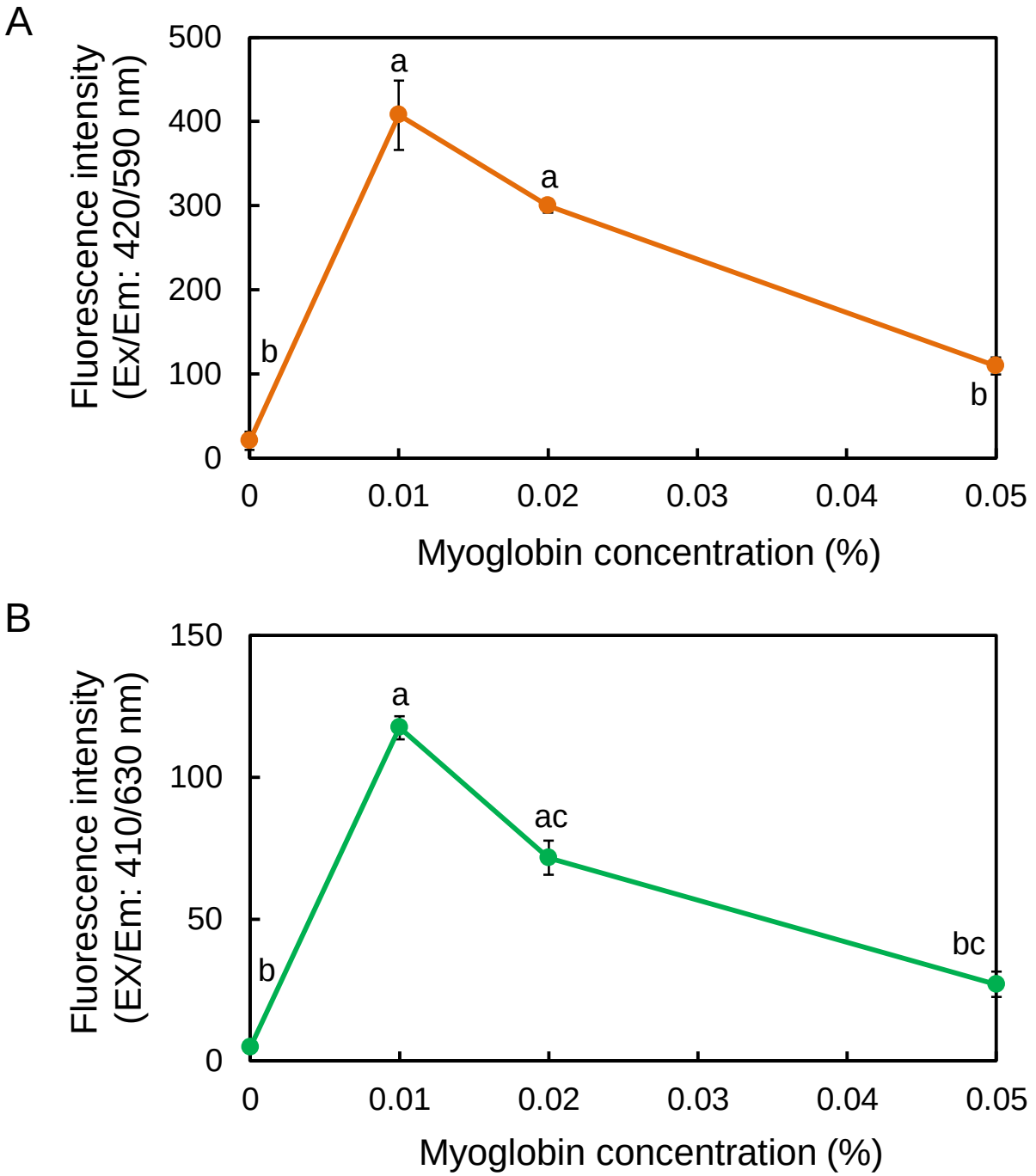


Fig. 13. Effect of exogenous oxymyoglobin on ZnPP (A) and PPIX (B) formation in IS muscle homogenate at pH 4.75.

Oxymyoglobin solution was added to the IS muscle homogenate in varying concentrations. The solution was incubated anaerobically for 5 days in the dark at 37°C with the addition of antibiotics and EDTA (for PPIX formation). ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

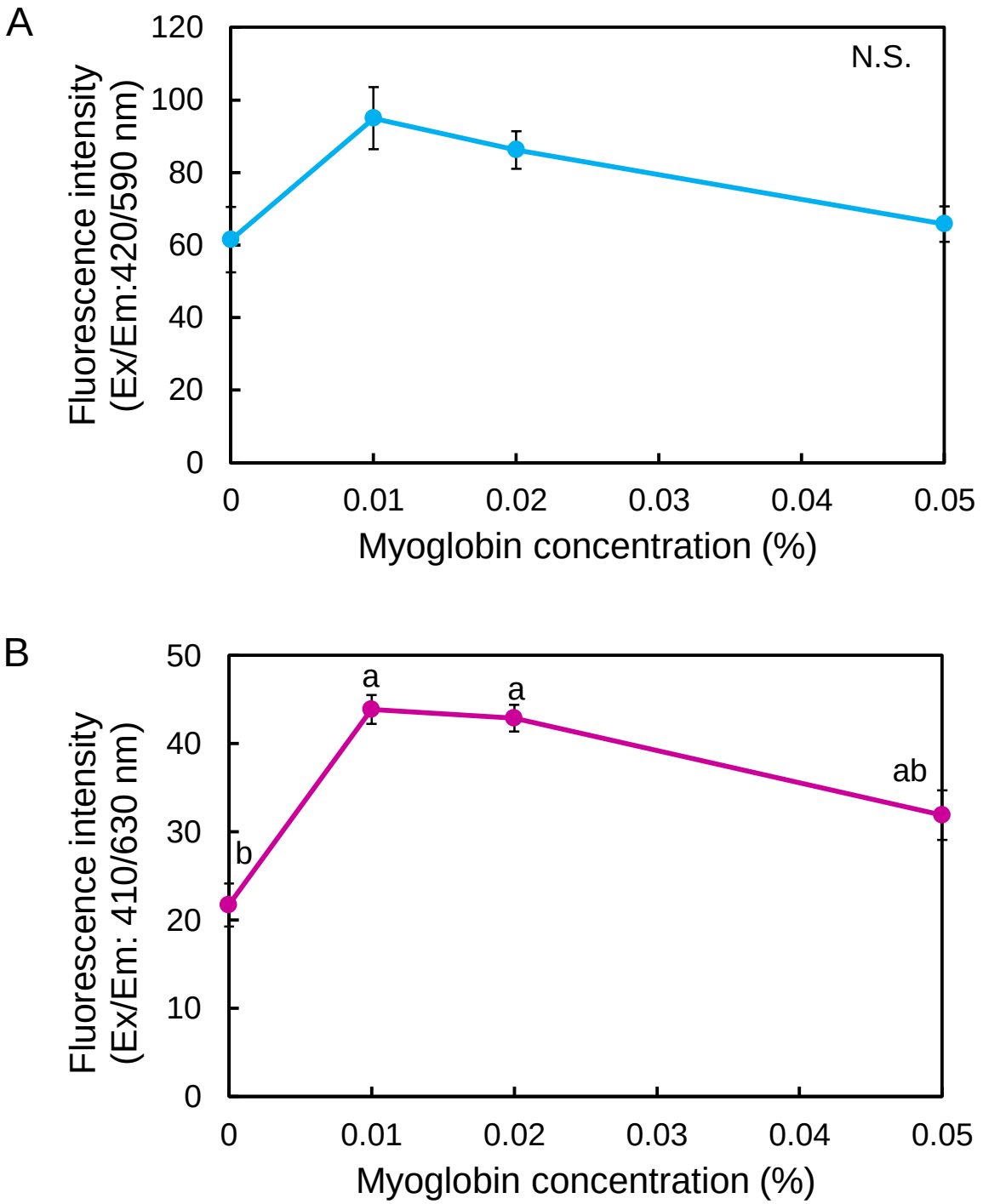


Fig. 14. Effect of impurities in myoglobin reagent on ZnPP (A) and PPIX (B) formation in IS muscle homogenate at pH 4.75.

Oxymyoglobin solution was subjected to ultrafiltration with a 10 kDa MWCO membrane to separate the impurities and the myoglobin. The impurities were added to the IS muscle homogenate and incubated with the addition of antibiotics and EDTA (for PPIX formation) anaerobically at 37°C for 5 days. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: Values in the same graph bearing different letters differ significantly ($P < 0.01$). N.S.: Non-significant.

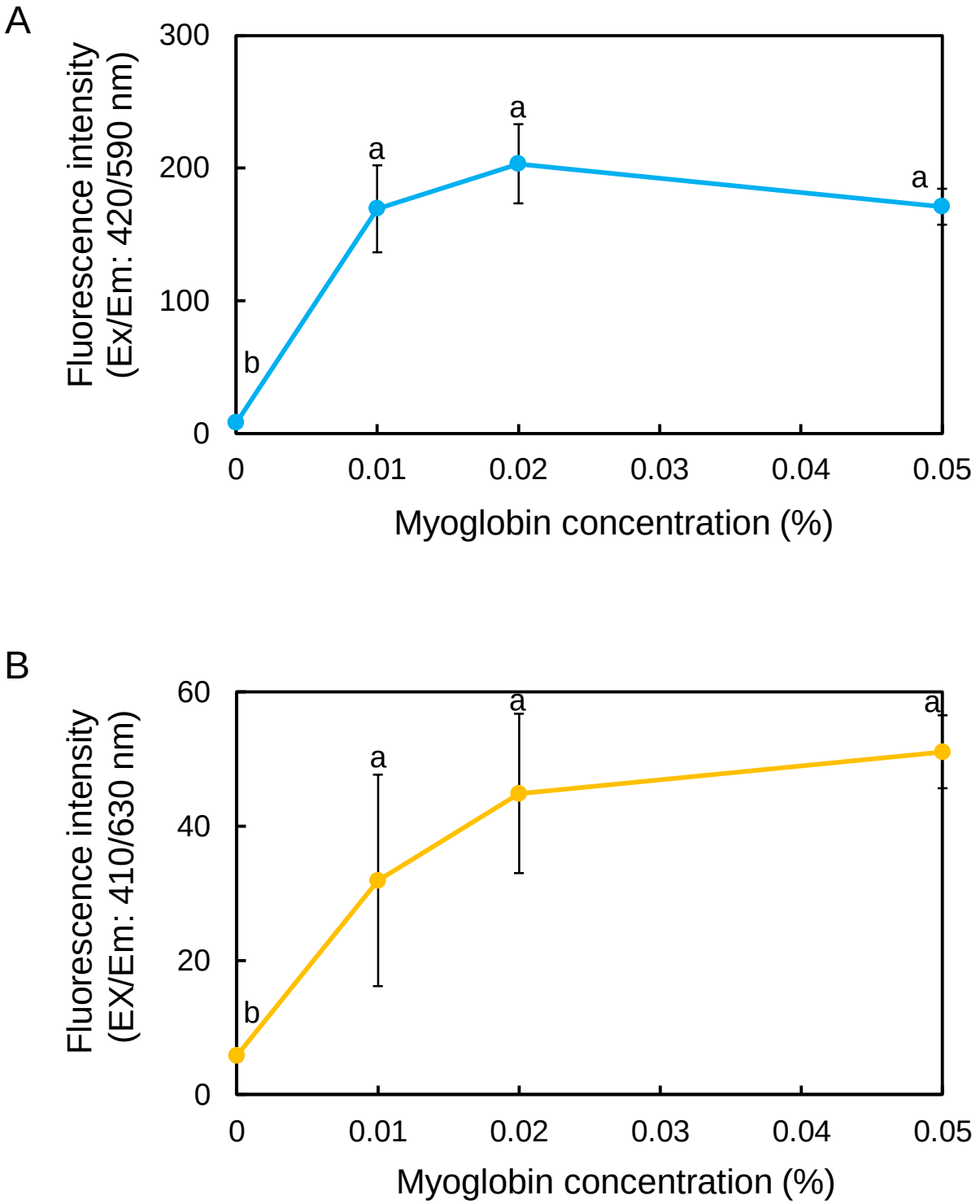


Fig. 15. Effect of exogenous desalted oxymyoglobin on ZnPP (A) and PPIX (B) formation in IS muscle homogenate at pH 4.75.

Desalted oxymyoglobin was added to the IS muscle homogenate. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically at 37°C for 5 days. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

3.1.7.2. Effect of exogenous myoglobin instead of >10 kDa soluble fraction

Next, the effect of exogenous oxymyoglobin instead of the >10 kDa soluble fraction on ZnPP and PPIX formation at pH 4.75 was investigated (Fig. 16). When oxymyoglobin was added at 0.01%, the amount of ZnPP (Fig. 16A) and PPIX (Fig. 16B) were significantly increased. But, when added in higher concentrations at 0.02 and 0.05%, the further increase of ZnPP and PPIX formation were not observed like the results in section 3.1.7.1. When the desalted oxymyoglobin was added to the <10 kDa soluble and insoluble fractions at the same concentration, the amount of ZnPP (Fig. 17A) and PPIX (Fig. 17B) formed were not increased linearly depending on the increased myoglobin concentration but were significantly increased with the addition of the desalted myoglobin. Since the exogenous myoglobin instead of the >10 kDa soluble fraction facilitate ZnPP and PPIX formation at pH 4.75, the endogenous myoglobin in the soluble fraction might contribute to the formation of ZnPP and PPIX at pH 4.75.

3.1.8. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by cation exchange chromatography

Next, in order to separate the water-soluble protein, the IS water-soluble fraction was subjected to cation exchange chromatography (CIEX). Since the pH of the model system using IS muscle homogenate was 4.75, the separation was performed at pH 4.75. After incubation of the CIEX-separated fractions with the insoluble and <10 kDa fractions, ZnPP (Fig. 18A) and PPIX (Fig. 18B) formation of the eluted fractions were significantly lower than the control. Next, the CIEX-separated fractions were incubated in any single or mixed groups with the addition of the insoluble and <10 kDa fractions (Fig. 19). The endogenous myoglobin was present in the unadsorbed fraction (0 M, data not shown). The results also showed that the amount of ZnPP (Fig. 19A) and PPIX (Fig.

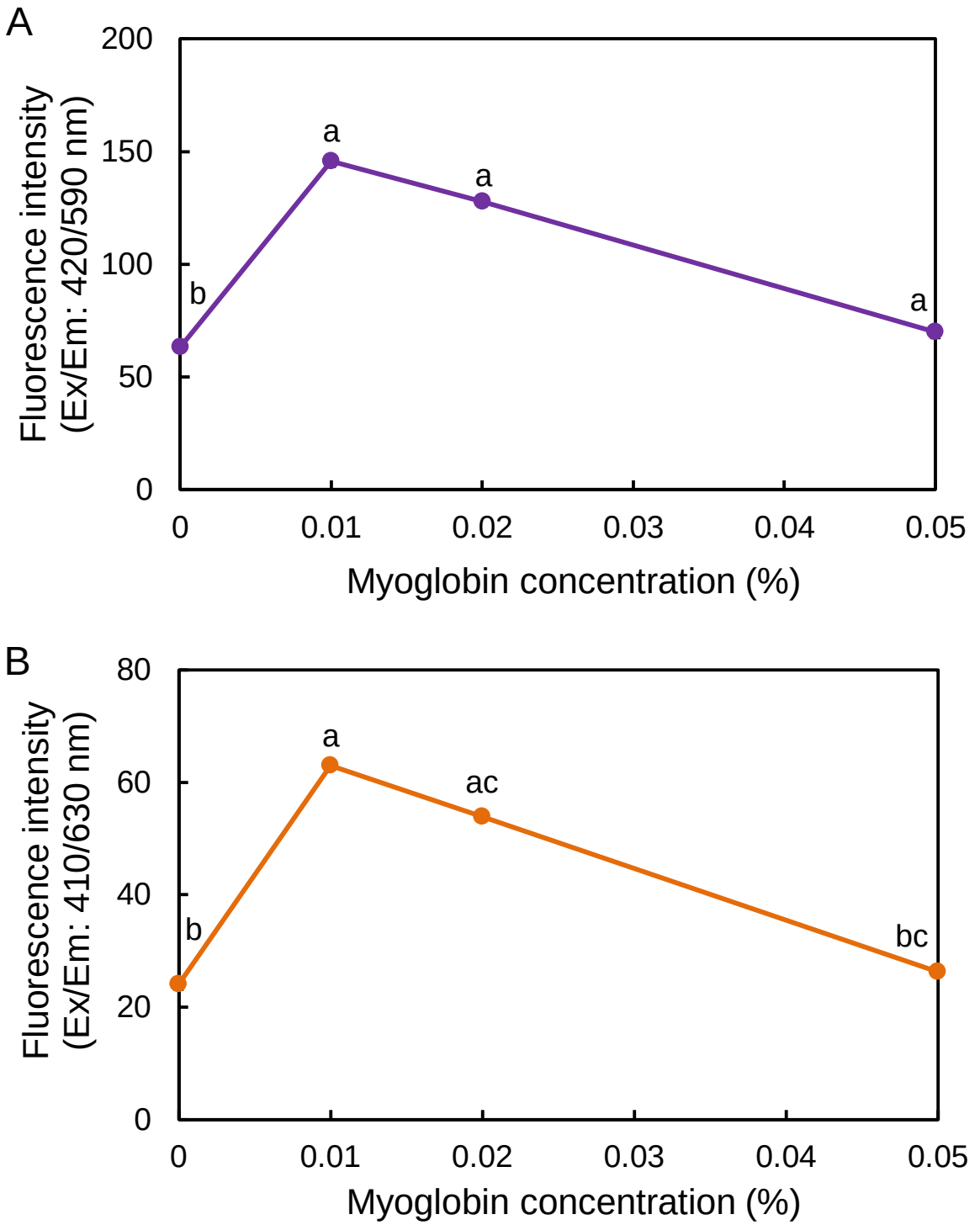


Fig. 16. Effect of exogenous oxymyoglobin instead of >10 kDa soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated IS muscle at pH 4.75.

Oxymyoglobin solution were added to the insoluble and <10 kDa soluble fractions with varying concentrations. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

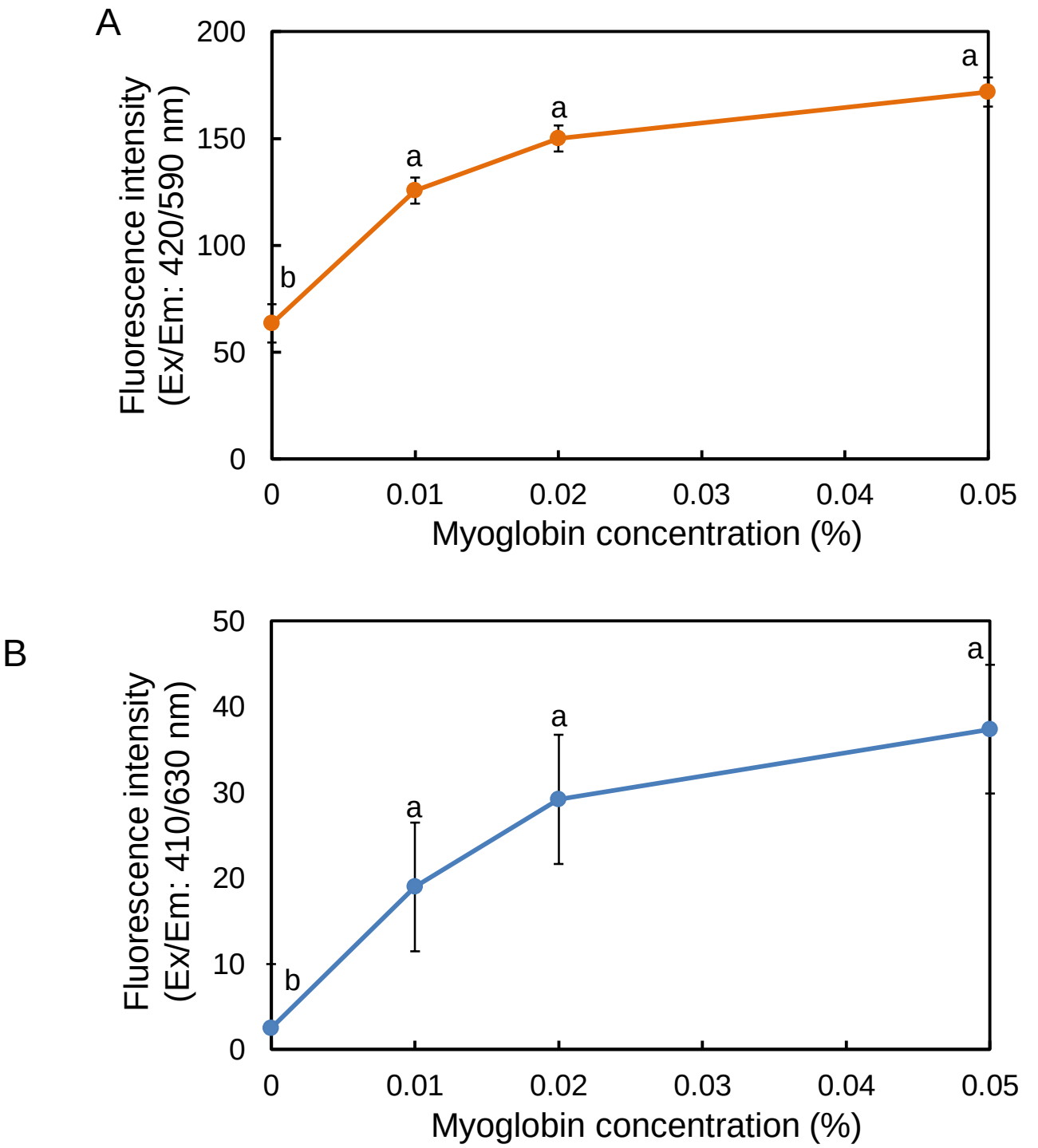


Fig. 17. Effect of exogenous desalted myoglobin instead of >10 kDa soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated IS muscle at pH 4.75.

Desalted oxymyoglobin was added to the insoluble and <10 kDa soluble fractions in different concentrations. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

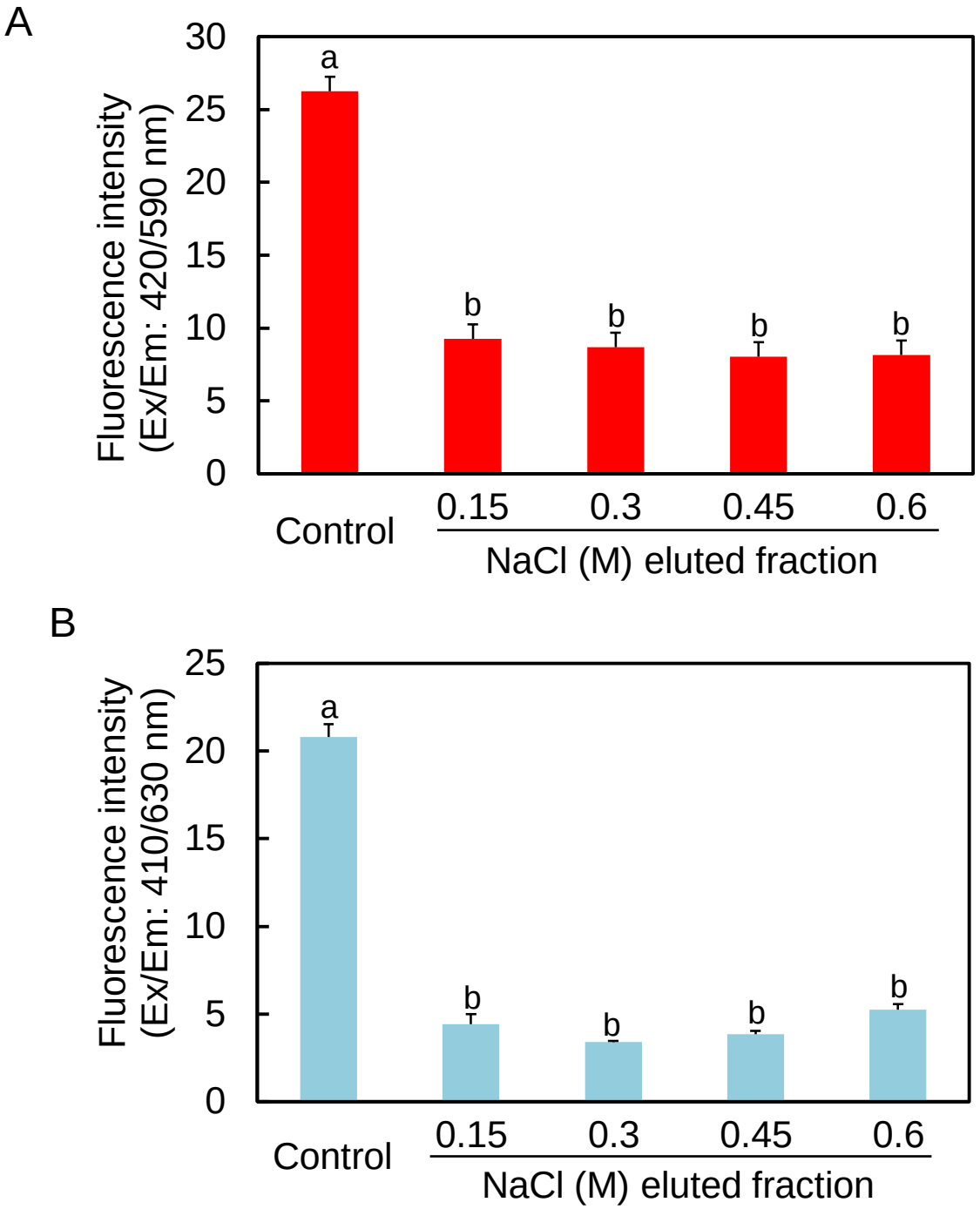


Fig. 18. ZnPP (A) and PPIX (B) forming ability at pH 4.75 of fractions eluted with different NaCl concentrations from CIEX column.

Water-soluble fraction (IS muscle) was subjected to cation exchange gel column (CM-Toyopearl 650M). The sample was first washed with 10 mM citrate buffer (pH 4.75), subsequently stepwise eluted with 0.15, 0.3, 0.45 and 0.6 M NaCl. The washed and eluted fractions were collected and mixed with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: Values bearing different letters differ significantly ($P < 0.01$).

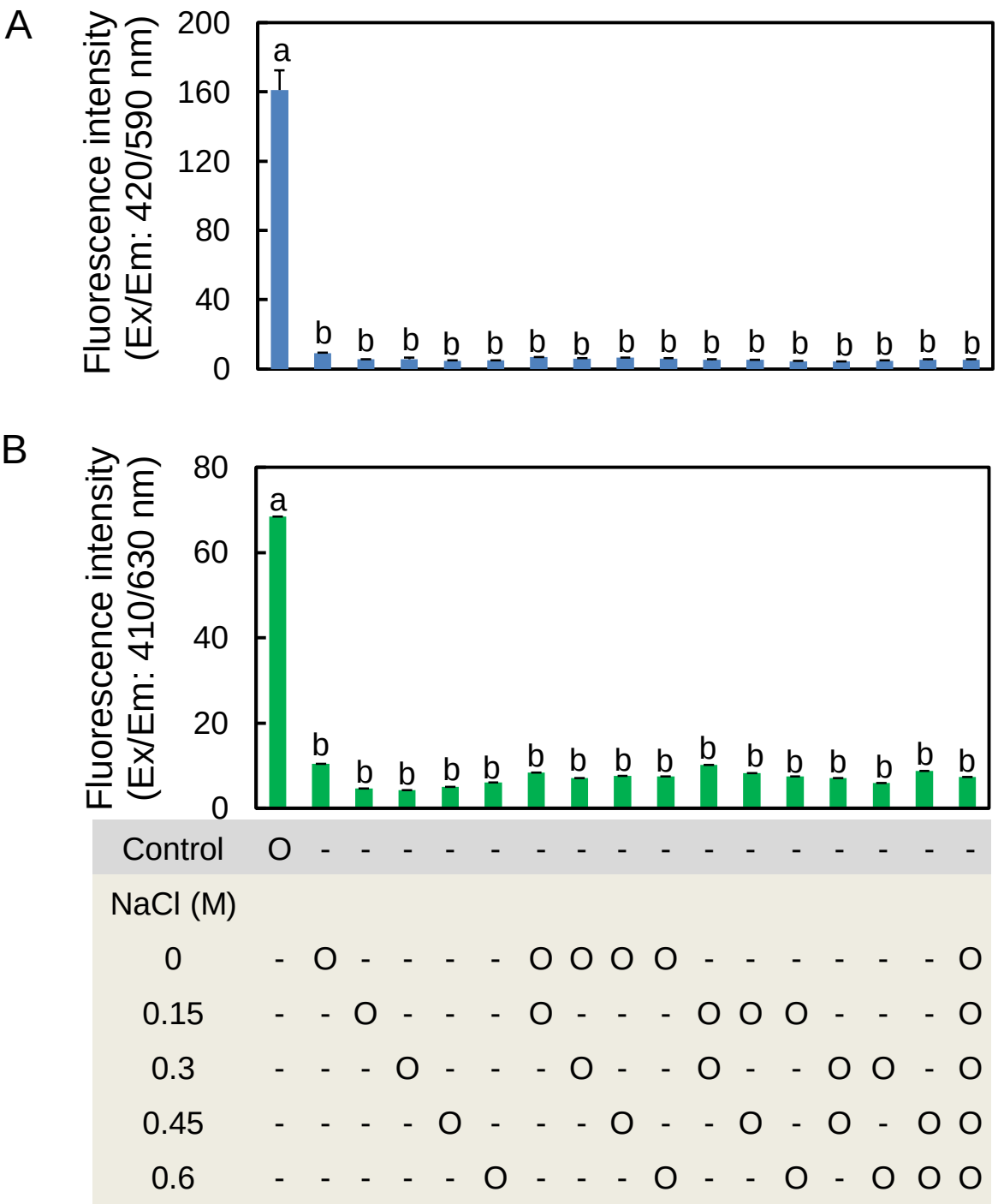


Fig. 19. Effect of combination of fractions eluted with different NaCl concentration from CIEX column on ZnPP (A) and PPIX (B) formation at pH 4.75.

IS water-soluble fraction was washed with 10 mM citrate buffer (pH 4.75), subsequently stepwise eluted with 0.15, 0.3, 0.45 and 0.6 M NaCl. The sample was concentrated and mixed with the insoluble and <10 kDa soluble fractions in single and mixed groups. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: values bearing different letters differ significantly ($P < 0.01$).

19B) formed were significantly lower in any single or mixed groups than that of control. Therefore, the contributor in the >10 kDa soluble fraction might be not only myoglobin. Moreover, the elution with NaCl from ion exchange chromatography might affect ZnPP and PPIX formation in the model experiment system at pH 4.75.

Next, in order to elute other contributors except myoglobin from CIEX column, the separation of the contributor proteins was performed by using a buffer with higher pH (up to 8.0). The water-soluble fraction was applied to a CIEX column equilibrated with buffer of each pH and the washing and collection with the same pH buffers (without NaCl) were performed. The unadsorbed fractions washed with higher pH buffers were incubated with the insoluble and <10 kDa soluble fractions. The amounts of ZnPP (Fig. 20A) formed was still significantly lower in all groups though the kinds of unadsorbed protein increased with the increase of pH by SDS-PAGE analysis (Fig. 21). In case of PPIX formation, a little increasing tendency was observed (Fig. 20B). The myoglobin was present in all pH buffer-washed fractions (data not shown). Therefore, soluble protein(s) other than myoglobin also might contribute to ZnPP and PPIX formation at pH 4.75 and was still bind strongly with the resin under high pH condition.

3.1.9. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by gel filtration chromatography at pH 4.75

Next, in order to investigate the contribution of endogenous myoglobin or other water-soluble protein(s) in the >10 kDa soluble fraction to ZnPP and PPIX formation, the protein(s) from the water-soluble fraction was separated by using a gel filtration chromatography and similarly applied to ZnPP and PPIX formation model experiment systems. The absorbance of the eluted protein at 280 nm and myoglobin at 400 nm were

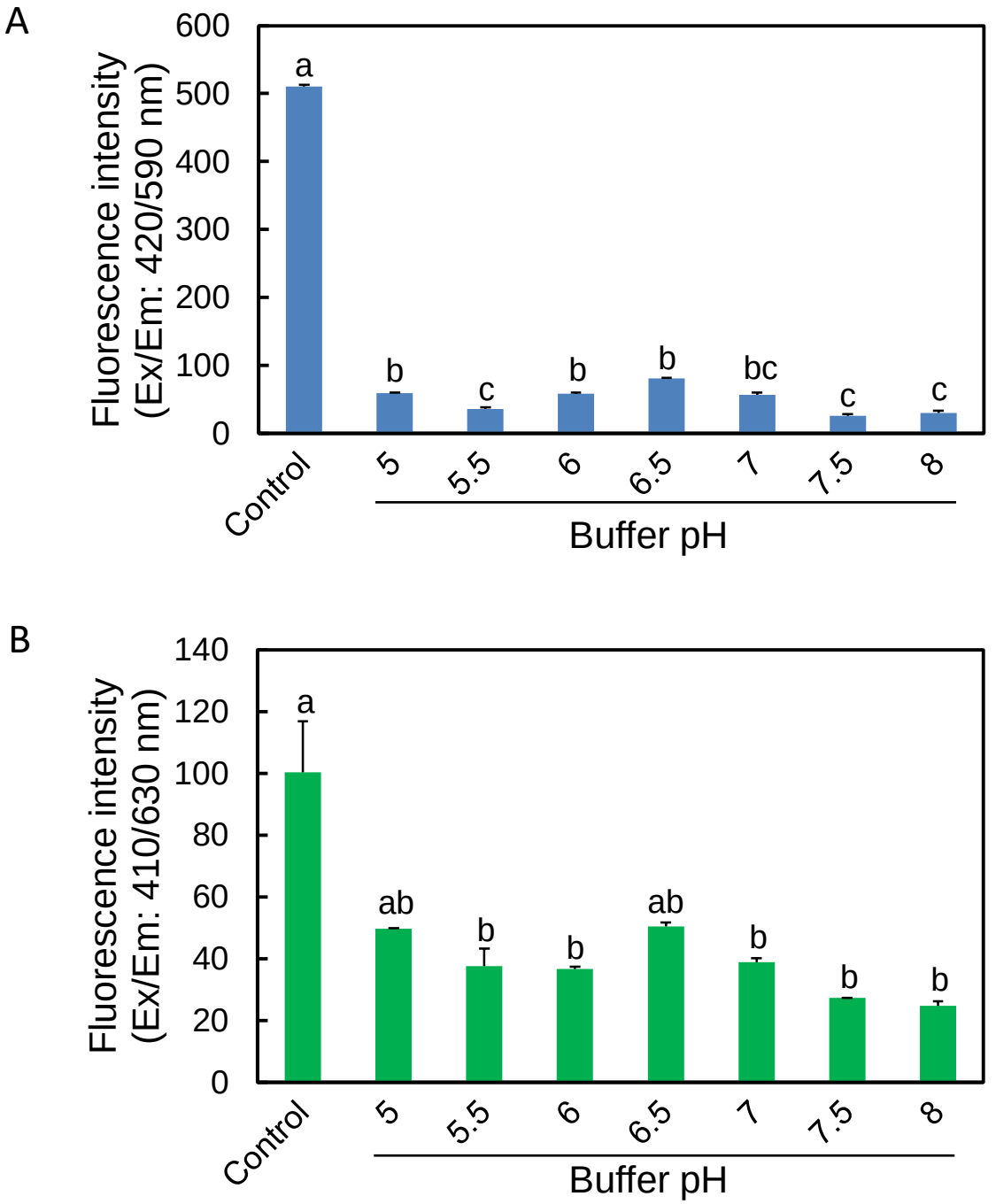


Fig. 20. ZnPP (A) and PPIX (B) forming ability at pH 4.75 of the unadsorbed fractions to a CIEX column at a various pH.

IS water-soluble fraction was applied to CIEX column equilibrated with 10 mM citrate buffer (pH 5.0-8.0). The unadsorbed fraction was collected and concentrated. Then, the concentrated fractions were mixed with the insoluble and <10 kDa soluble fractions separately. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 2). abc: values bearing different letters differ significantly ($P < 0.05$).

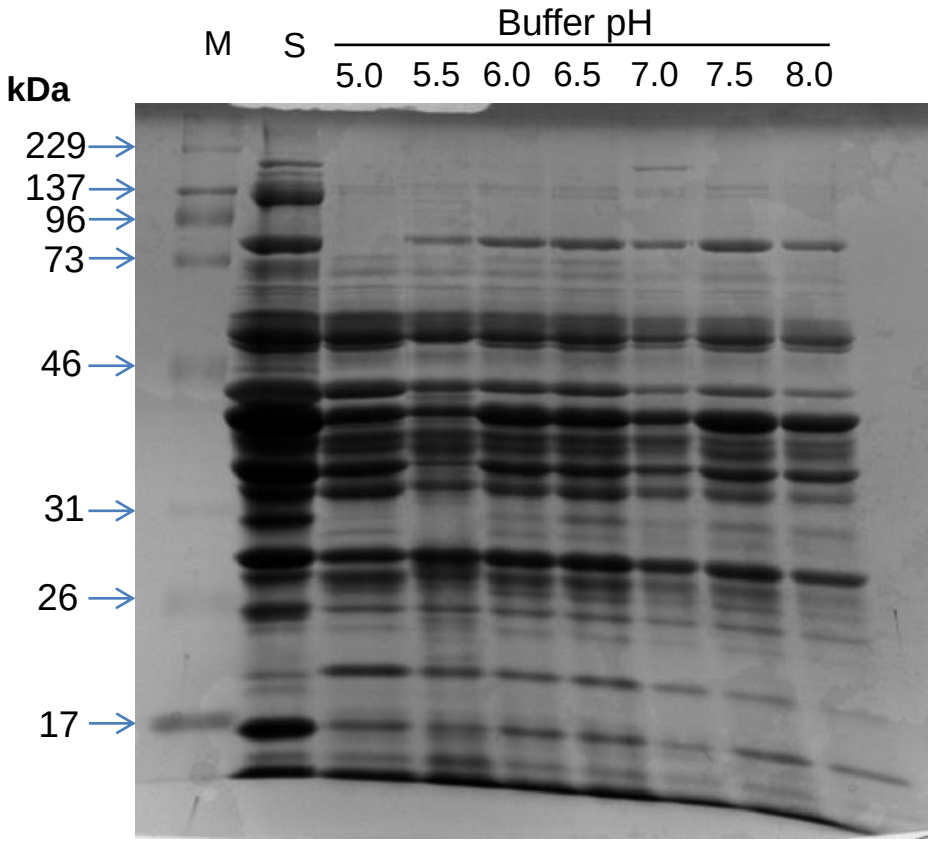


Fig. 21. SDS-PAGE image of fractions washed with different pH buffer from CIEX column.

SDS-PAGE of each washed fraction (Fig. 20) was performed using 4.5% acrylamide for the stacking gel and 15% acrylamide for the separating gel. The gel was stained with coomassie brilliant blue (CBB). M: Marker, S: Soluble fraction of IS muscle homogenate.

measured and SDS-PAGE of the separated fractions was performed (Fig. 22). When the separated fractions were incubated with the insoluble and <10 kDa soluble fractions separately, high ZnPP and PPIX were formed with the myoglobin eluted fractions and ZnPP and PPIX formation were optimum in myoglobin absorbance peak (Fig. 23). In SDS-PAGE image, the band of myoglobin was observed in high ZnPP and PPIX-forming fractions (Fig. 22B).

As suggested in section 3.1.6., the water-soluble protein with molecular weight of 10-30 kDa has a high ability to form ZnPP and PPIX. Therefore, 10-30 kDa soluble fraction was separated by gel filtration chromatography and similarly applied to ZnPP and PPIX formation model experiment systems. The number of main protein peaks was reduced due to the removal of high molecular protein by ultrafiltration (Fig. 24). The myoglobin-containing fractions still indicated high abilities to form ZnPP (Fig. 24A) and PPIX (Fig. 24B) like above. When the Pearson correlation coefficient between ZnPP/PPIX formation and the myoglobin contents of the separated fractions at pH 4.75 was calculated, the amount of ZnPP (Fig. 25A) and PPIX (Fig. 25B) formed were significantly correlated with the myoglobin contents. Therefore, the water-soluble protein with molecular weight close to that of myoglobin might contribute to ZnPP and PPIX formation at pH 4.75.

3.2. Mechanism of ZnPP formation in LD muscle at pH 5.5

It was reported that PPIX is a precursor for the formation of ZnPP at pH 5.5 (Wakamatsu et al., 2007) and fractionation of pork is advantageous to search for the components contributing to ZnPP formation (Shiraishi, 2010). However, the components responsible for ZnPP formation are still unknown. To elucidate ZnPP/PPIX formation mechanism at pH 5.5, this research separated the LD muscle homogenate and

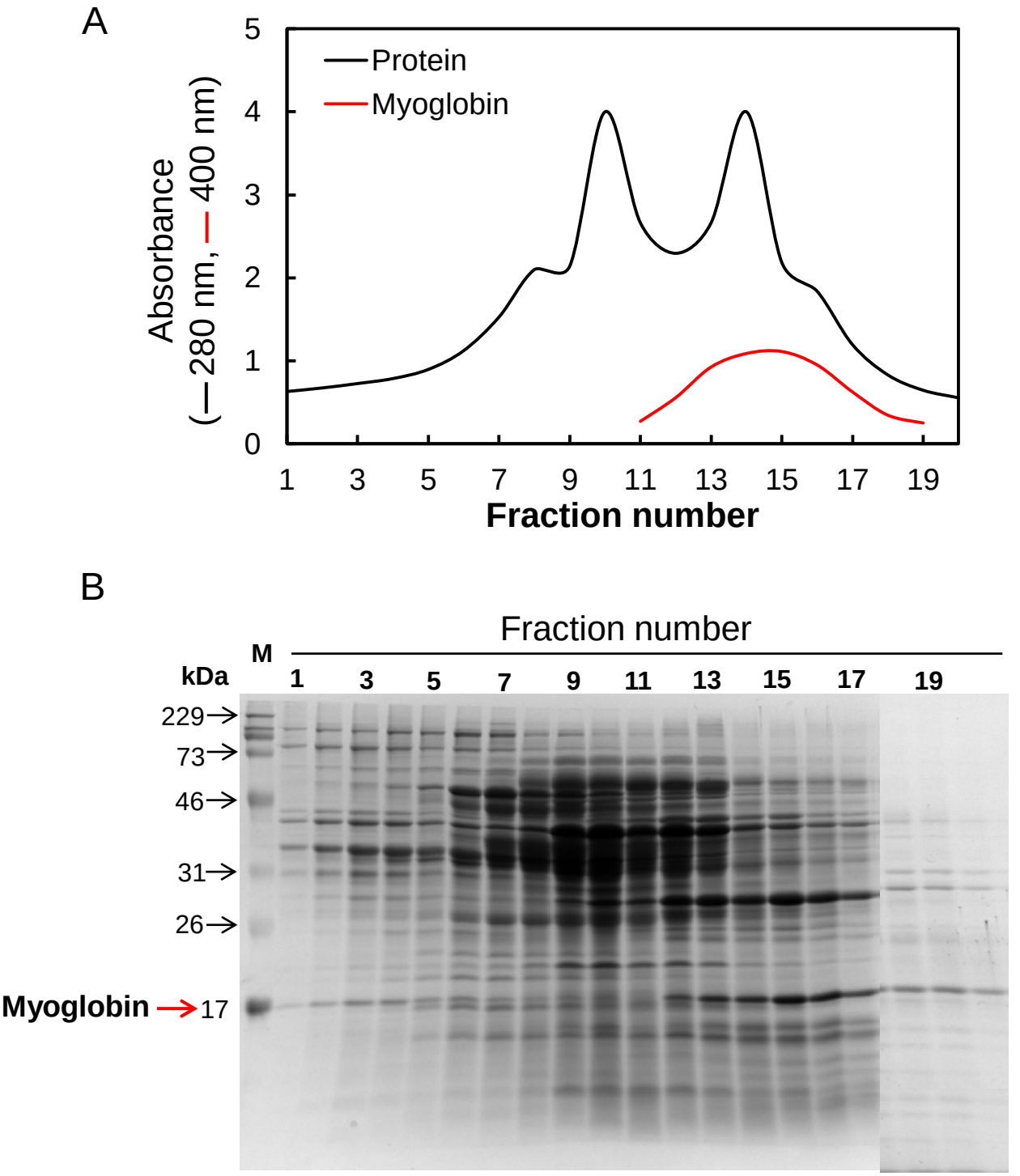


Fig. 22. Gel filtration chromatogram (A) of IS water-soluble fraction and SDS-PAGE image (B) of eluted fractions in IS muscle at pH 4.75.

A) Concentrated >10 kDa soluble fraction was subjected to the gel filtration chromatography (Toyopearl HW 55F, 3.3 φ × 60 cm). Flow rate was 0.25 ml/min and each fraction contained 5 ml sample. The absorbance was measured at 280 nm and 400 nm for protein and myoglobin content, respectively.

B) SDS-PAGE of each fraction was performed using 4.5% acrylamide of stacking gel and 15% acrylamide of separating gel and stained with CBB. M: Marker.

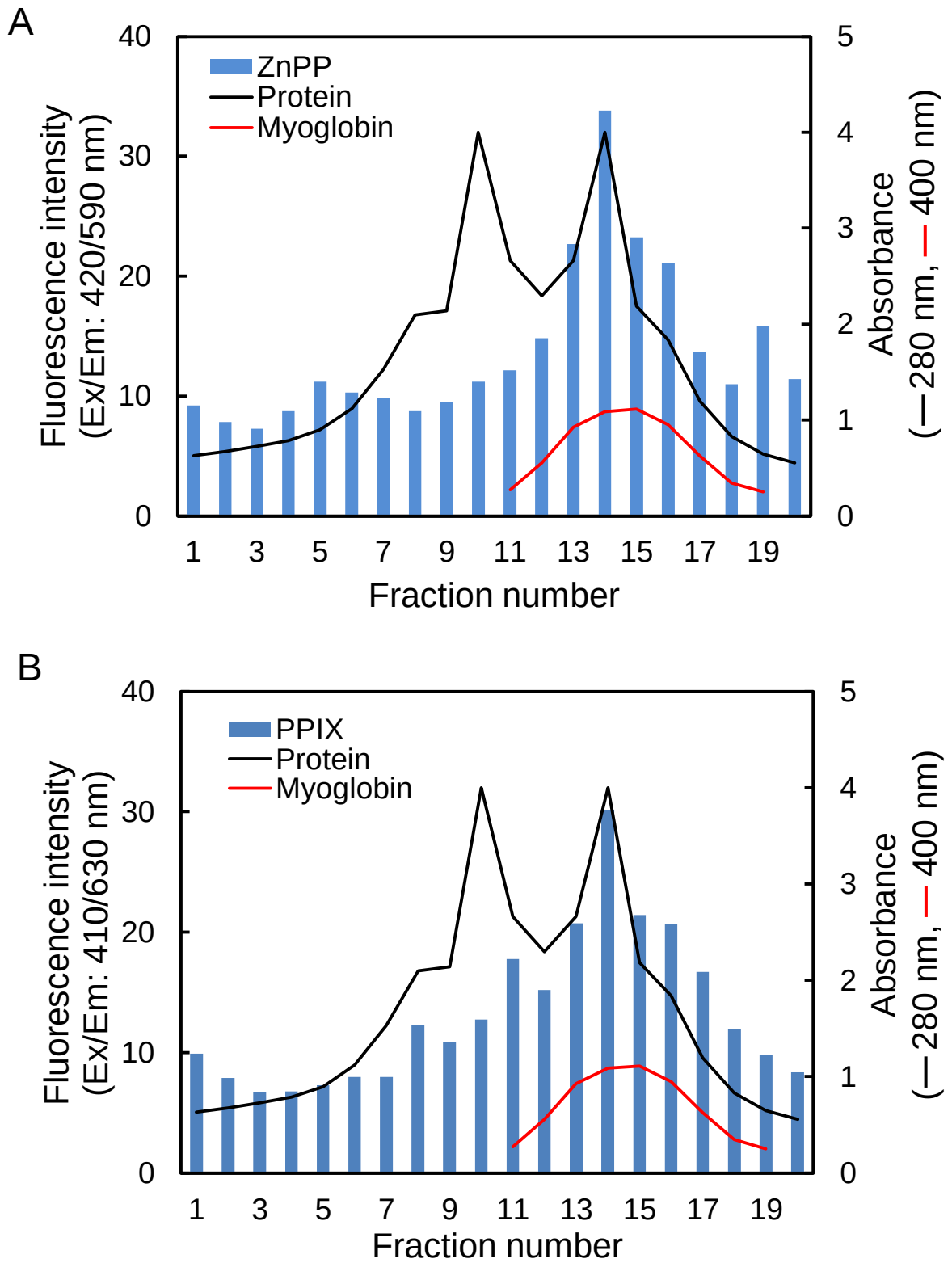


Fig. 23. ZnPP (A) and PPIX (B) forming ability of gel filtrated fractions of IS water-soluble fractions.

Each eluted fraction (Fig. 22A) was incubated with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured.

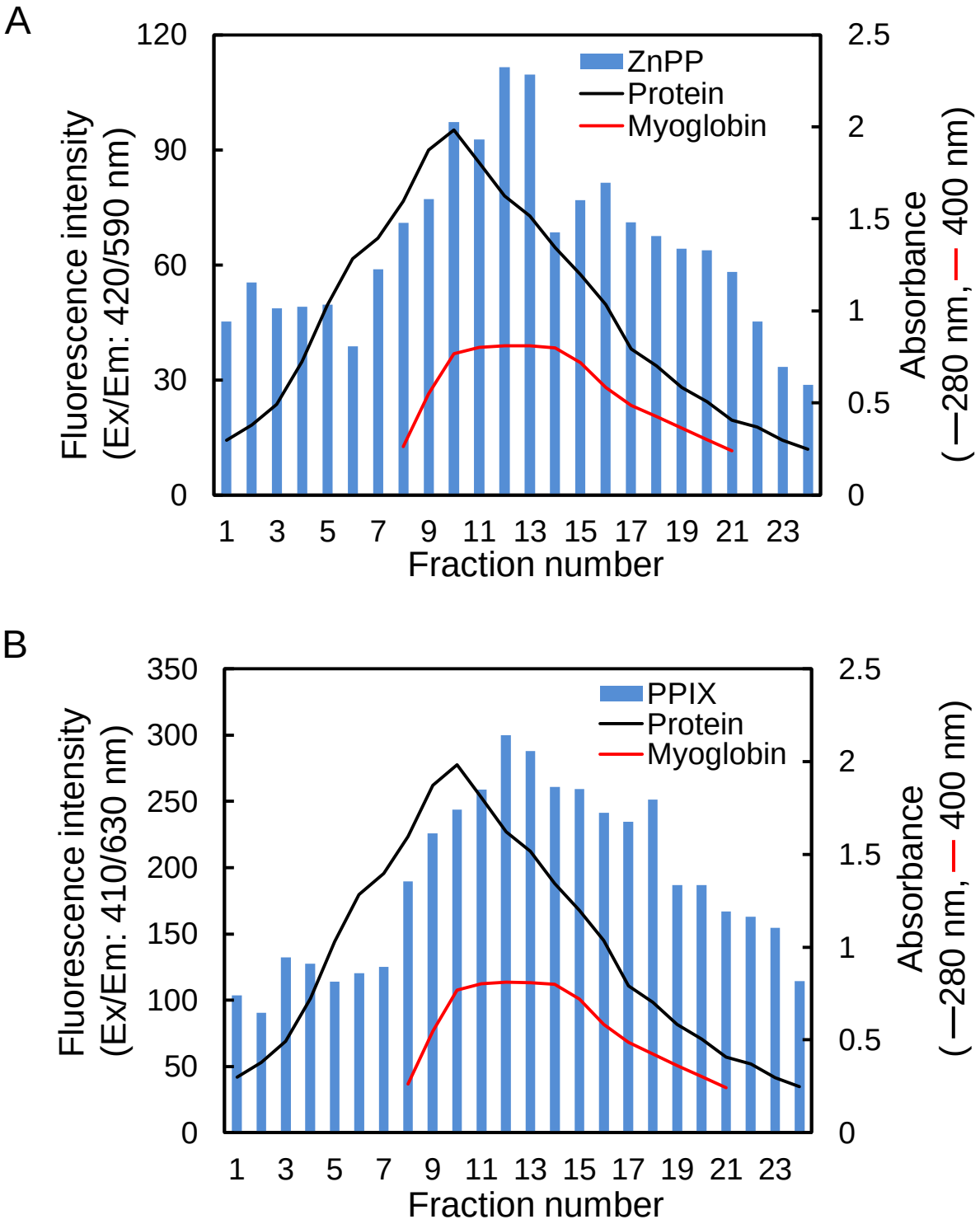


Fig. 24. ZnPP (A) and PPIX (B) forming ability of gel filtrated fractions of IS 10-30 kDa soluble fraction.

Concentrated 10-30 kDa water-soluble fraction was applied to the gel filtration chromatography. Flow rate was 0.25 ml/min. The absorbance was measured for protein and myoglobin. After the addition of antibiotics, each eluted fraction was incubated with the insoluble and <10 kDa soluble fractions for 5 days anaerobically in the dark at 37°C. ZnPP and PPIX were extracted separately and the fluorescence intensity was measured.

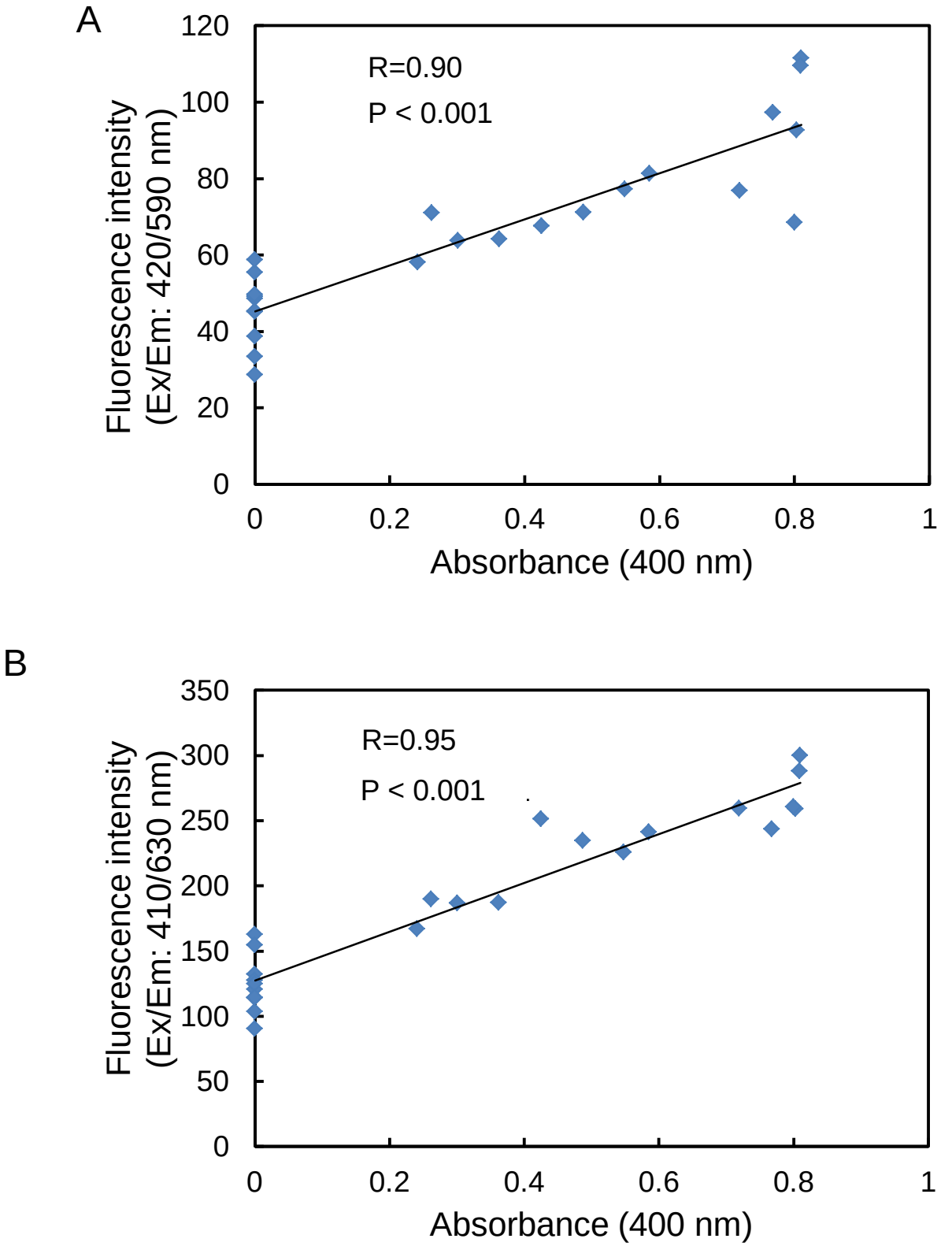


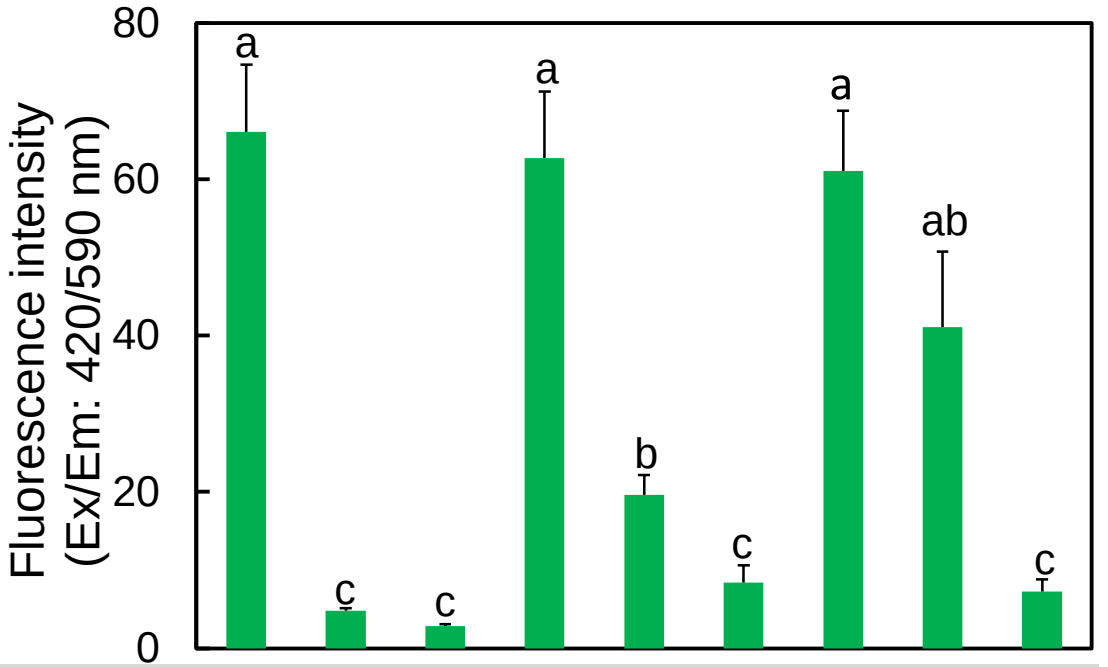
Fig. 25. Correlation between ZnPP (A) and PPIX (B) formation at pH 4.75 and the myoglobin content of the gel filtrated fractions.

Pearson correlation coefficient was calculated between ZnPP & PPIX formation and the myoglobin absorbance of the separated fractions from Fig. 24. R= Pearson correlation coefficient.

checked ZnPP and PPIX formation in the separated fraction. Furthermore, the water-soluble protein of LD muscle was separated by using various chromatography techniques and the role of myoglobin was examined in ZnPP/PPIX formation model experiment systems. Since ZnPP/PPIX formation was optimum at pH 5.5 in LD muscle (Wakamatsu et al., 2007), LD muscle was used in this section.

3.2.1. Effect of fractionation on ZnPP and PPIX formation

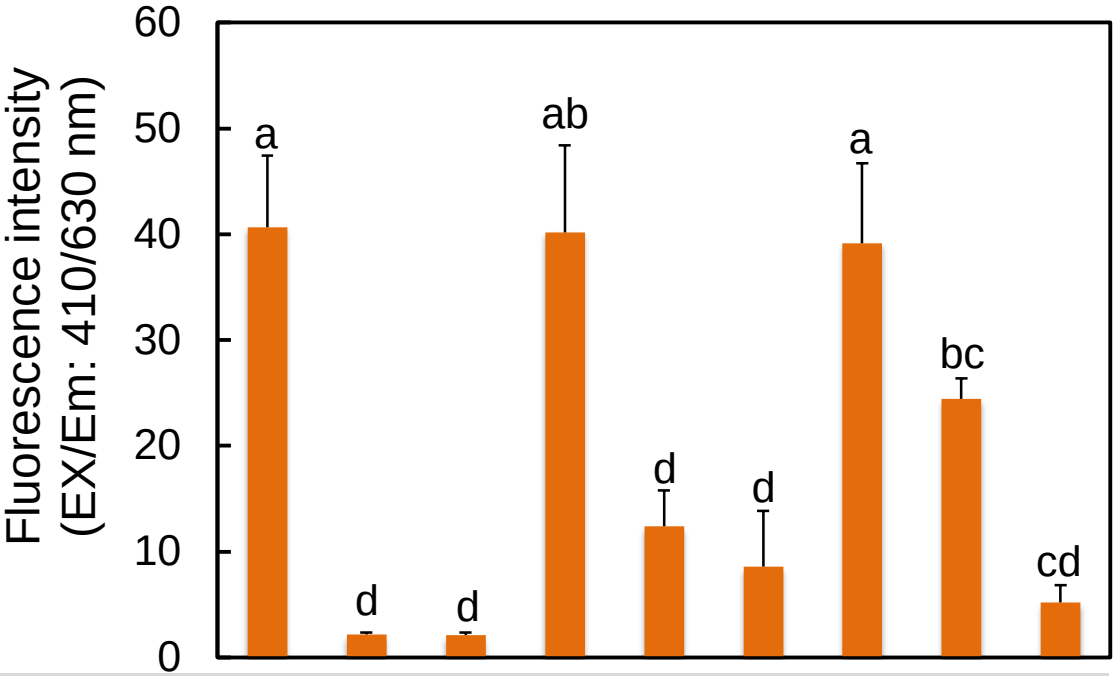
First, to check the contributors to ZnPP and PPIX formation at pH 5.5, LD muscle homogenate was fractionated into different fractions and the effect of separated fractions on ZnPP (Fig. 26) and PPIX (Fig. 27) formation at pH 5.5 was investigated. When LD homogenate was fractionated into the insoluble and soluble fractions and incubated individually, ZnPP formation was significantly suppressed (Fig. 26). When they were mixed together, the formation of ZnPP was rescued. When the soluble fraction was further fractionated into the >10 and <10 kDa soluble fractions by ultrafiltration and the separated fractions were incubated with the insoluble fraction separately, ZnPP formation was significantly suppressed. When the >10 and <10 kDa soluble fractions were mixed together with the insoluble fraction, ZnPP formation was rescued as LD muscle homogenate. As regards PPIX formation by the fractionated LD muscle homogenate, the same behaviors were observed similarly to ZnPP formation (Fig. 27). Therefore, this study suggested that one or more components in each fraction from LD muscle homogenate are essential for the formation of ZnPP as well as PPIX at pH 5.5.



Pork homogenate	O	-	-	-	-	-	-	-	-
Insoluble fraction	-	-	O	O	O	O	O	O	O
Soluble fraction	-	O	-	O	-	-	-	-	-
>10 kDa	-	-	-	-	O	-	O	O	-
<10 kDa	-	-	-	-	-	O	O	-	O
Heated >10 kDa	-	-	-	-	-	-	-	-	O
Heated <10 kDa	-	-	-	-	-	-	-	O	-

Fig. 26. Effect of fractionation on ZnPP formation in LD muscle at pH 5.5.

LD muscle homogenate (pH 5.5) was separated into the water-soluble and insoluble fractions. The water-soluble fraction was separated into the >10 and <10 kDa soluble fractions and heated separately. After the addition of antibiotics to single and mixed fractions, it was incubated anaerobically in dark for 5 days at 25°C. ZnPP was extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values bearing different letters differ significantly ($P < 0.05$).



Pork homogenate	O	-	-	-	-	-	-	-	-
Insoluble fraction	-	-	O	O	O	O	O	O	O
Soluble fraction	-	O	-	O	-	-	-	-	-
>10 kDa	-	-	-	-	O	-	O	O	-
<10 kDa	-	-	-	-	-	O	O	-	O
Heated >10 kDa	-	-	-	-	-	-	-	-	O
Heated <10 kDa	-	-	-	-	-	-	-	O	-

Fig. 27. Effect of fractionation on PPIX formation in LD muscle at pH 5.5.

LD muscle homogenate was separated into the water-soluble and insoluble fractions. The water-soluble fraction was separated into the >10 and <10 KDa soluble fractions and heated separately. After the addition of EDTA and antibiotics to single and mixed fractions, it was incubated anaerobically in the dark for 5 days at 37°C. PPIX was extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abcd: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

3.2.2. Effect of heat treatment of fractionated water-soluble fraction on ZnPP and PPIX formation

Next, similarly to section 3.1.5., the heating effect of the >10 kDa soluble fraction was examined on ZnPP (Fig. 26) and PPIX (Fig. 27) formation at pH 5.5. When the heated >10 kDa soluble fraction of LD muscle was incubated with the insoluble and <10 kDa soluble fractions, ZnPP formation was significantly suppressed (Fig. 26). In case of PPIX formation, a similar result was observed (Fig. 27). Therefore, this study suggested that water-soluble protein presents in the >10 kDa soluble fraction contributed to ZnPP as well as PPIX formation at pH 5.5.

Then, the heating effect of the <10 kDa soluble fraction on ZnPP (Fig. 26) and PPIX (Fig. 27) formation was also examined at pH 5.5. When the heated <10 kDa soluble fraction was incubated with the insoluble and >10 kDa soluble fractions, the formation of ZnPP was not so suppressed. A similar finding was obtained in case of PPIX (Fig. 28). Thus, it was suggested that the heat-stable component(s) in the <10 kDa water-soluble fraction contributed to the formation of both ZnPP and PPIX at pH 5.5.

3.2.3. Effect of separation of water-soluble fraction according to molecular weight on ZnPP and PPIX formation

In the last section, it was revealed that water-soluble protein(s) is essential for the formation of ZnPP and PPIX at pH 5.5. Therefore, in order to investigate the molecular weight of the contributing protein, next the LD water-soluble fraction was subjected to ultrafiltration and their ZnPP and PPIX-forming abilities were investigated (Fig. 28). After separation of the <10, <30, <50 and <100 kDa soluble fractions, the separated fractions were incubated individually with the insoluble fraction. Although ZnPP (Fig.

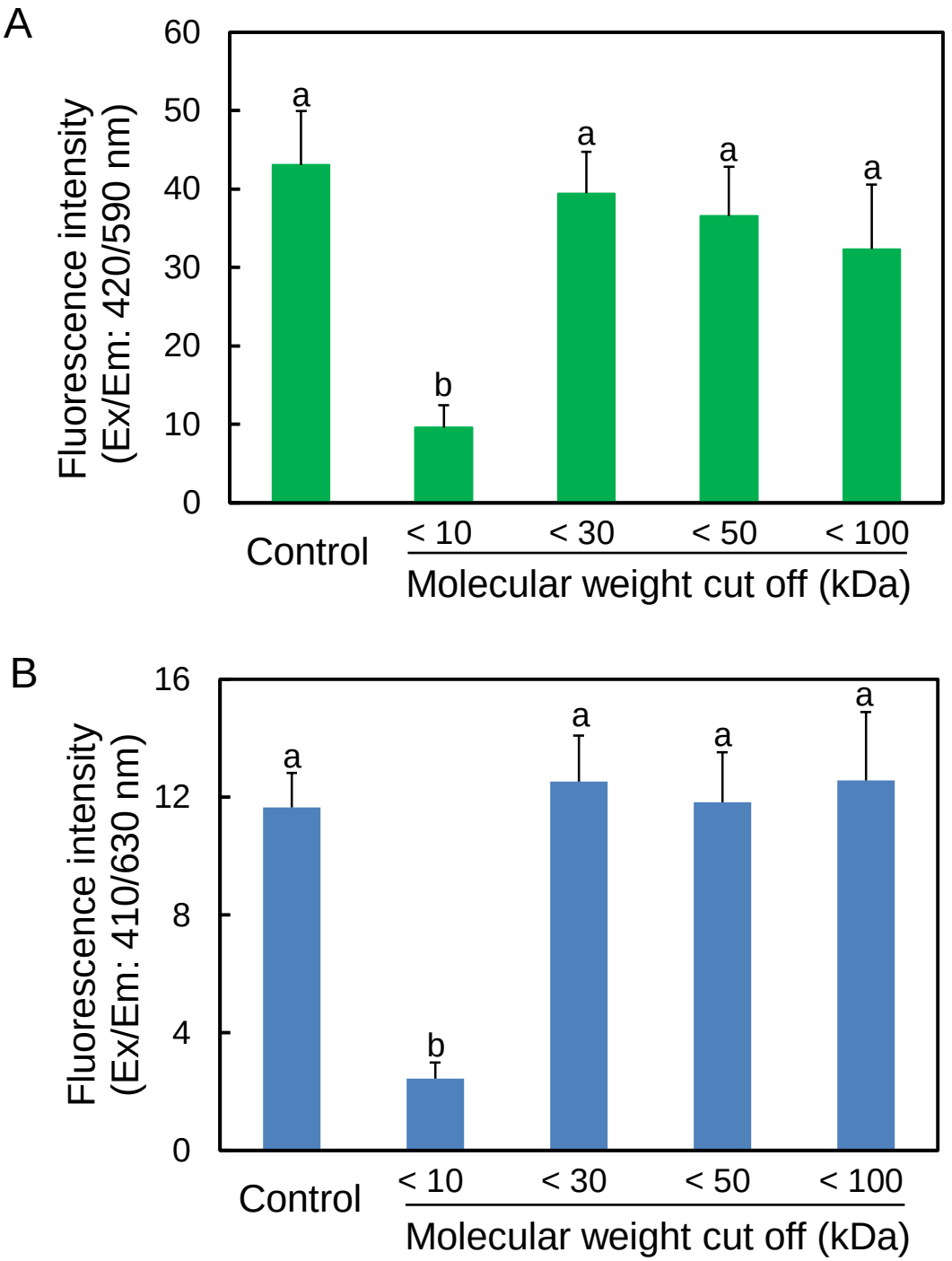


Fig. 28. Effect of ultrafiltration of water-soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated LD muscle at pH 5.5.

The water-soluble fraction of LD muscle homogenate (pH 5.5) was subjected to ultrafiltration through a molecular weight cut off 10, 30, 50 and 100 kDa ultrafiltration spin column. The filtrate was mixed with the insoluble fraction. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: Values in the same graph bearing different letters differ significantly ($P < 0.05$).

28A) and PPIX (Fig. 28B) formation were suppressed in the <10 kDa soluble fraction group, it was rescued in the <30 kDa soluble fraction group as well as control. Since the amounts of ZnPP and PPIX formed in the <50 and <100 kDa soluble fraction groups did not increase compared with the <30 kDa fraction group, the >30 kDa soluble protein(s) might not contribute to the formation of ZnPP and PPIX. Thus, the present study suggested that water-soluble protein(s) having molecular weight 10-30 kDa is significant to the formation of ZnPP and PPIX at pH 5.5.

Next, the additional effect of the <10 kDa soluble fraction on ZnPP and PPIX formation at pH 5.5 was observed in the separated molecular weight groups (Fig. 29). After the addition of the extra <10 kDa soluble fractions to the groups (<30, <50 and <100 kDa) with the insoluble fraction, the amount of ZnPP and PPIX were not significantly increased. The <30, <50 and <100 kDa soluble fractions contain the sufficient amount of the <10 kDa fractions for ZnPP and PPIX formation. Therefore, the addition of the extra <10 kDa soluble fraction might not beneficial to ZnPP and PPIX formation at pH 5.5.

3.2.4. Effect of exogenous myoglobin on ZnPP and PPIX formation

In order to verify the involvement of myoglobin on ZnPP and PPIX formation like at pH 4.75, the effect of myoglobin on ZnPP and PPIX formation at pH 5.5 were studied using commercially available myoglobin reagent.

3.2.4.1. Effect of exogenous myoglobin on ZnPP and PPIX formation

From above, the effects of myoglobin on ZnPP and PPIX formation in LD muscle homogenate at pH 5.5 was investigated (Fig. 30). The result showed that the addition of oxymyoglobin at 0.01, 0.02 and 0.05% in LD muscle homogenate slightly inhibited the

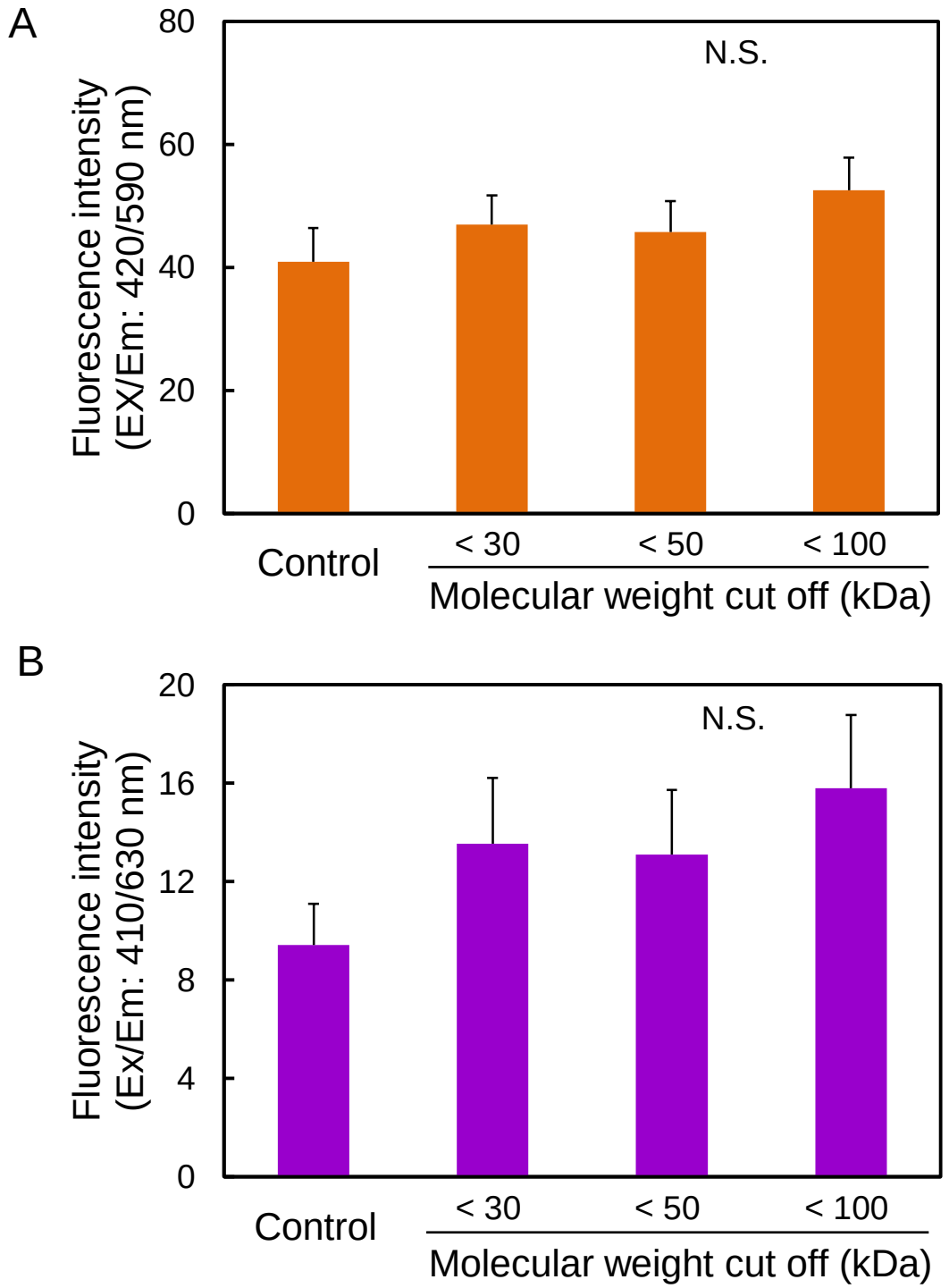


Fig. 29. Supplemental effect of <10 kDa soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated LD muscle at pH 5.5.

The water-soluble fraction of pork homogenate (pH 5.5) was subjected to ultrafiltration through a molecular weight cut off 30, 50 and 100 kDa ultrafiltration spin column. Each filtrate was mixed with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). N.S.: Non-significant.

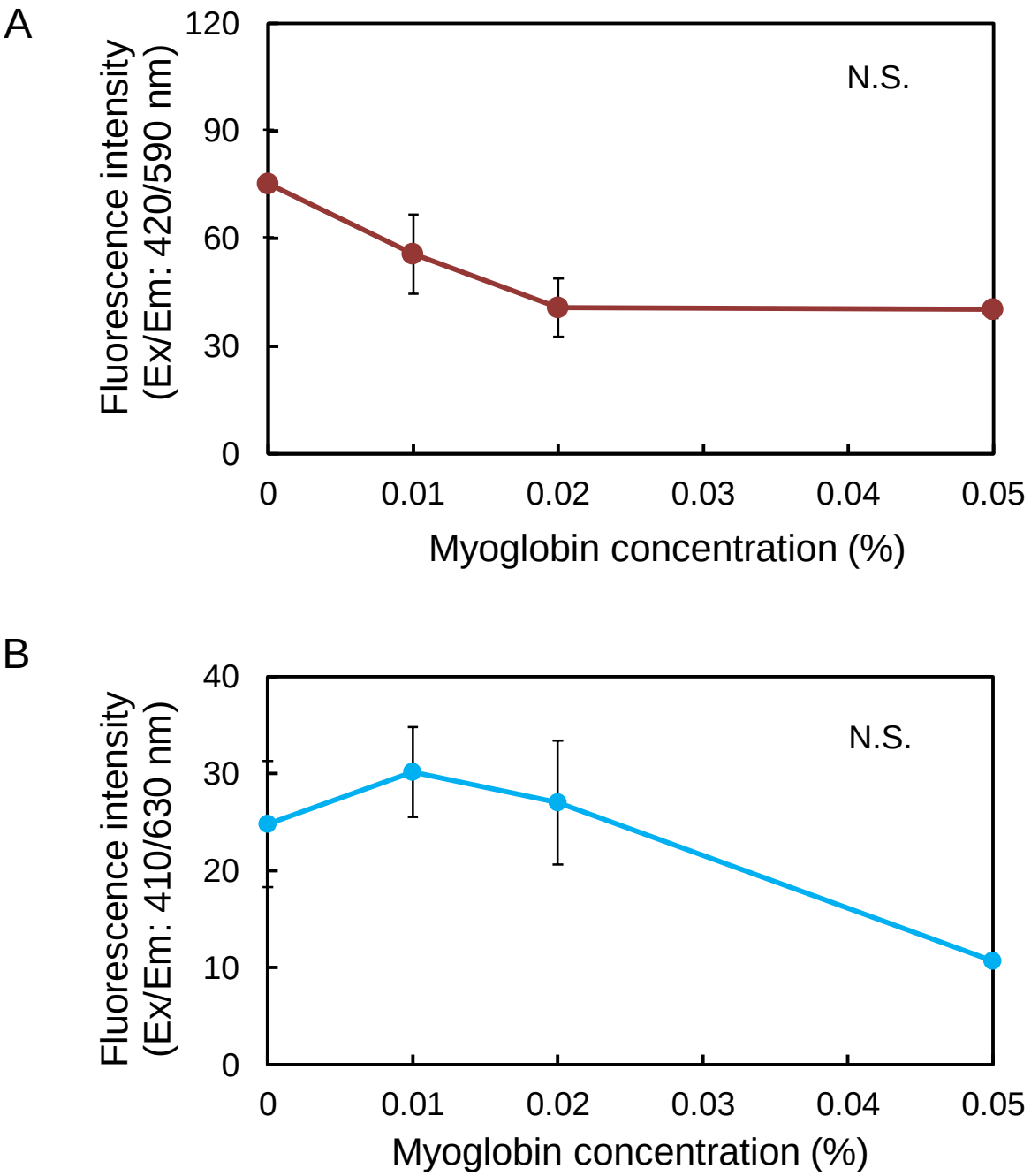


Fig. 30. Effect of exogenous oxymyoglobin on ZnPP (A) and PPIX (B) formation in LD muscle homogenate at pH 5.5.

Oxymyoglobin solution was added to the LD muscle homogenate (pH 5.5). The solution was incubated with the addition of antibiotics and EDTA (for PPIX formation) anaerobically in the dark for 5 days at 37°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). N.S.: Non-significant.

ZnPP formation (Fig. 30A). On the other hand, 0.01% oxymyoglobin slightly increased PPIX formation little but further addition decreased PPIX formation (Fig. 30B). Next, the impurities were separated from oxymyoglobin solution by ultrafiltration and also applied to ZnPP and PPIX formation model experiment systems (Fig. 31). The addition of impurities to LD homogenate inhibited ZnPP (Fig. 31A) and PPIX (Fig. 31B) formation but there are no significant differences. When desalted oxymyoglobin was added to LD homogenate at the same concentration, the amount of ZnPP (Fig. 32A) and PPIX (Fig. 32B) formed were increased compared with the non-myoglobin group but there is no significant difference among them. Therefore, these results also suggested that exogenous myoglobin is not essential for the formation of ZnPP and PPIX formation in LD muscle homogenate at pH 5.5. In addition, the impurities of reagent myoglobin solution inhibited the formation of ZnPP and PPIX at pH 5.5.

3.2.4.2. Effect of exogenous myoglobin instead of >10 kDa soluble fraction

First, the supplemental effect of exogenous oxymyoglobin instead of the >10 kDa soluble fraction was investigated on ZnPP and PPIX formation at pH 5.5 (Fig. 33). When oxymyoglobin was added at 0.01% to the <10 kDa soluble and insoluble fractions of LD homogenate at pH 5.5, the amount of ZnPP and PPIX were increased. But, when added in higher concentrations at 0.02 and 0.05% of oxymyoglobin, the amount of ZnPP and PPIX formed amount were decreased. Similarly, when desalted oxymyoglobin was added in the same concentration to the <10 kDa soluble and insoluble fractions, the amount of ZnPP (Fig. 34A) and PPIX (Fig. 34B) formed were not significantly increased compared with the control. Therefore, these results supported that the component other than myoglobin in the <10 kDa soluble fraction contribute to ZnPP and PPIX formation at pH 5.5.

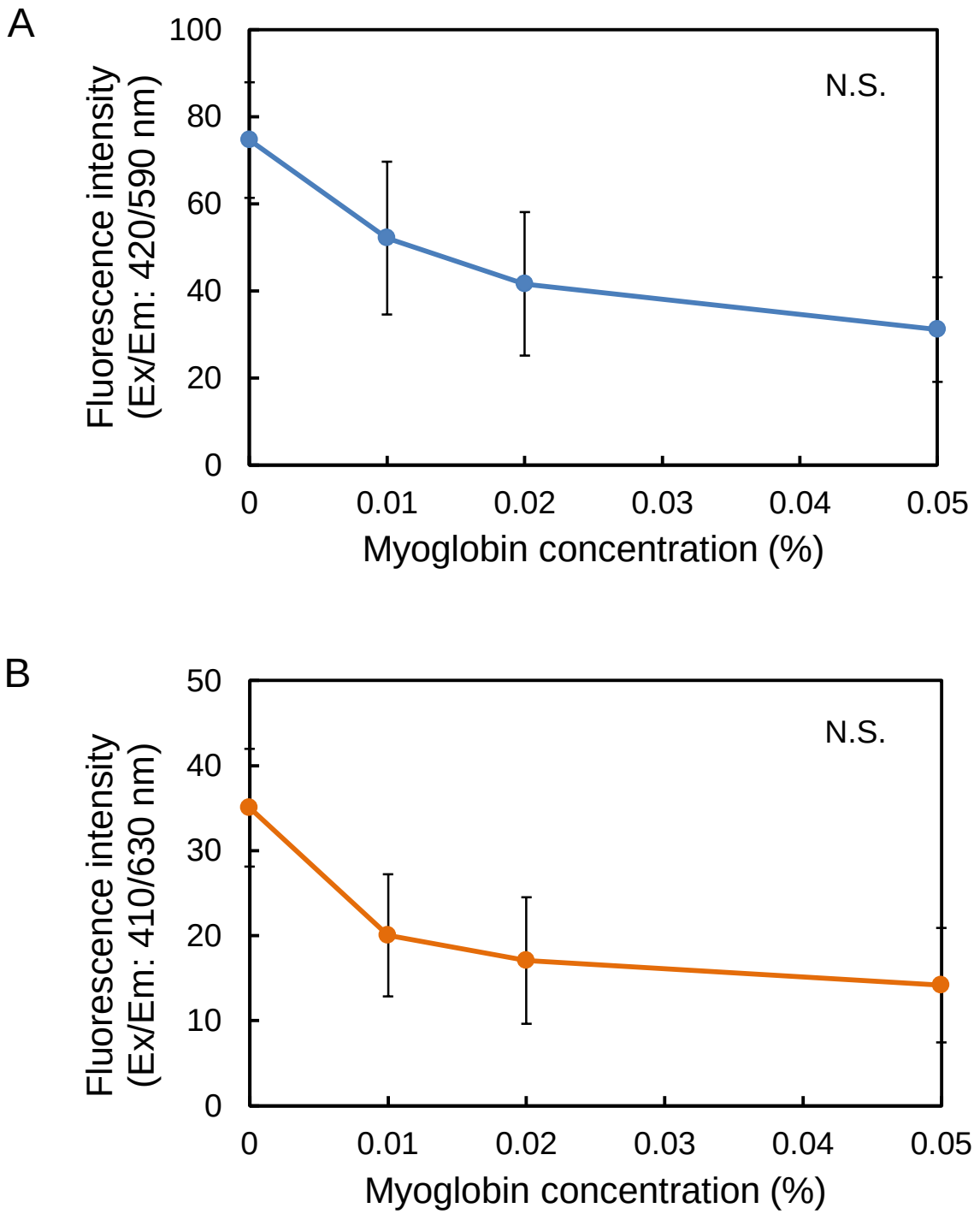


Fig. 31. Effect of impurities in myoglobin reagent on ZnPP (A) and PPIX (B) formation in LD muscle homogenate at pH 5.5.

Oxymyoglobin solution was subjected to ultrafiltration with a 10 kDa MWCO membrane to separate the impurities and myoglobin. The impurities were added to the LD muscle homogenate and incubated with the addition of antibiotics and EDTA (for PPIX formation) anaerobically in the dark at 25°C for 5 days. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). N.S.: Non-significant.

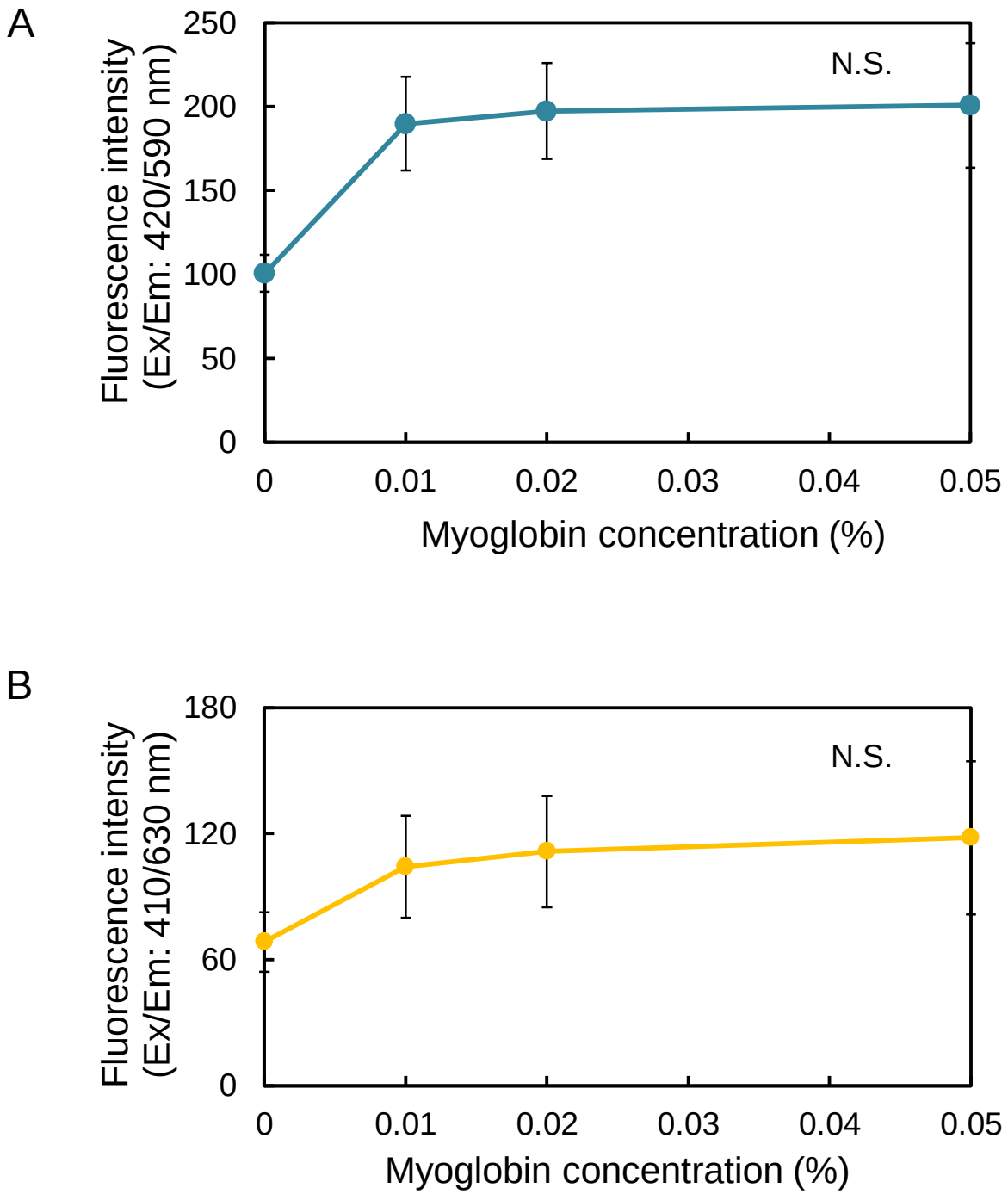


Fig. 32. Effect of exogenous desalted myoglobin ZnPP (A) and PPIX (B) formation in LD muscle homogenate on at pH 5.5.

Desalted oxymyoglobin was added to the LD muscle homogenate. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically at 25°C in the dark for 5 days. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). N.S.: Non-significant.

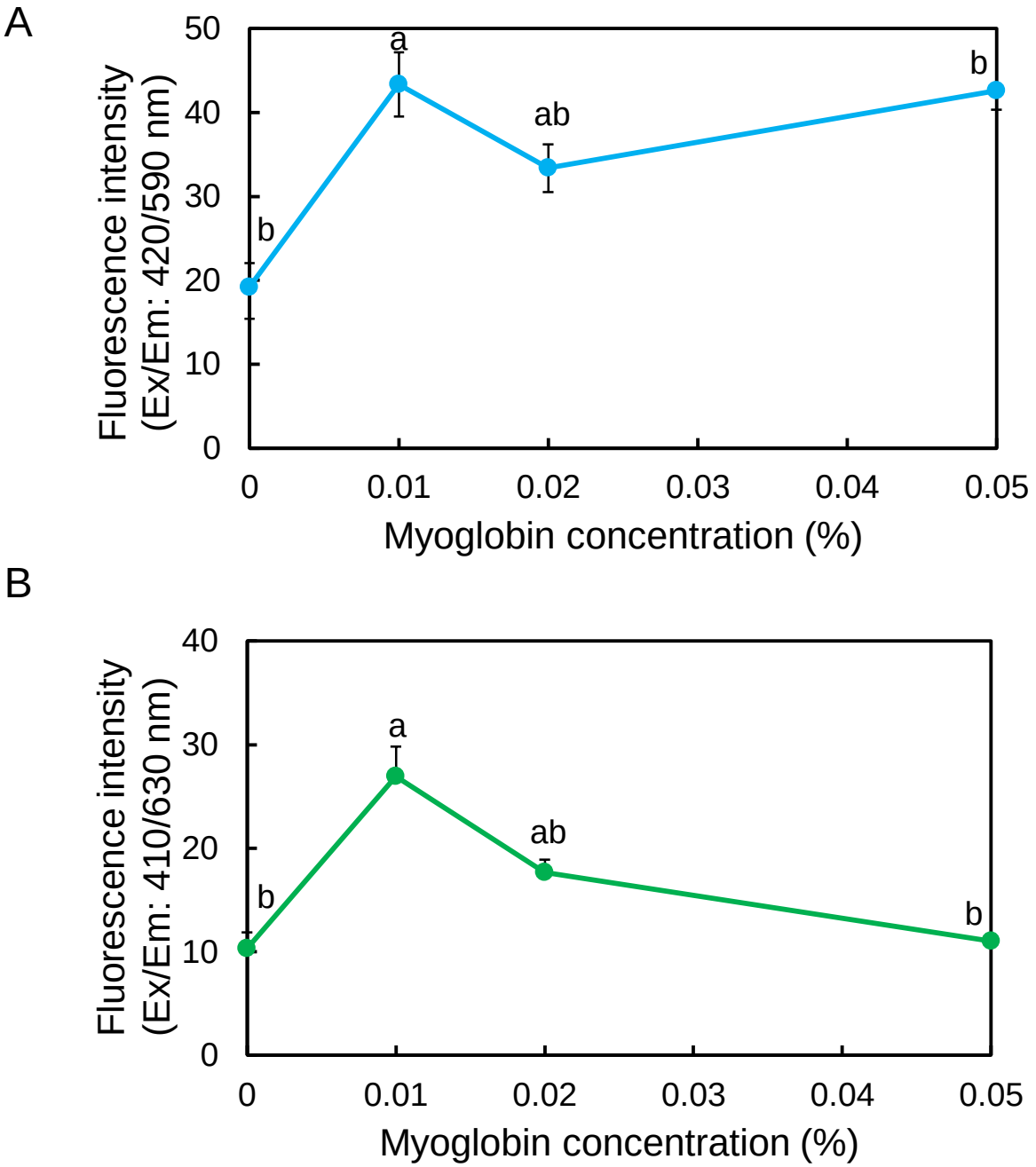


Fig. 33. Effect of exogenous oxymyoglobin instead of >10 kDa soluble fraction on ZnPP (A) and PPIX (B) formation in fractionated LD muscle at pH 5.5.

Oxymyoglobin solution was added to the LD insoluble and <10 kDa soluble fractions with varying concentrations. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bar represent the standard error of the means (n = 3). ab: Values bearing different letters differ significantly ($P < 0.01$).

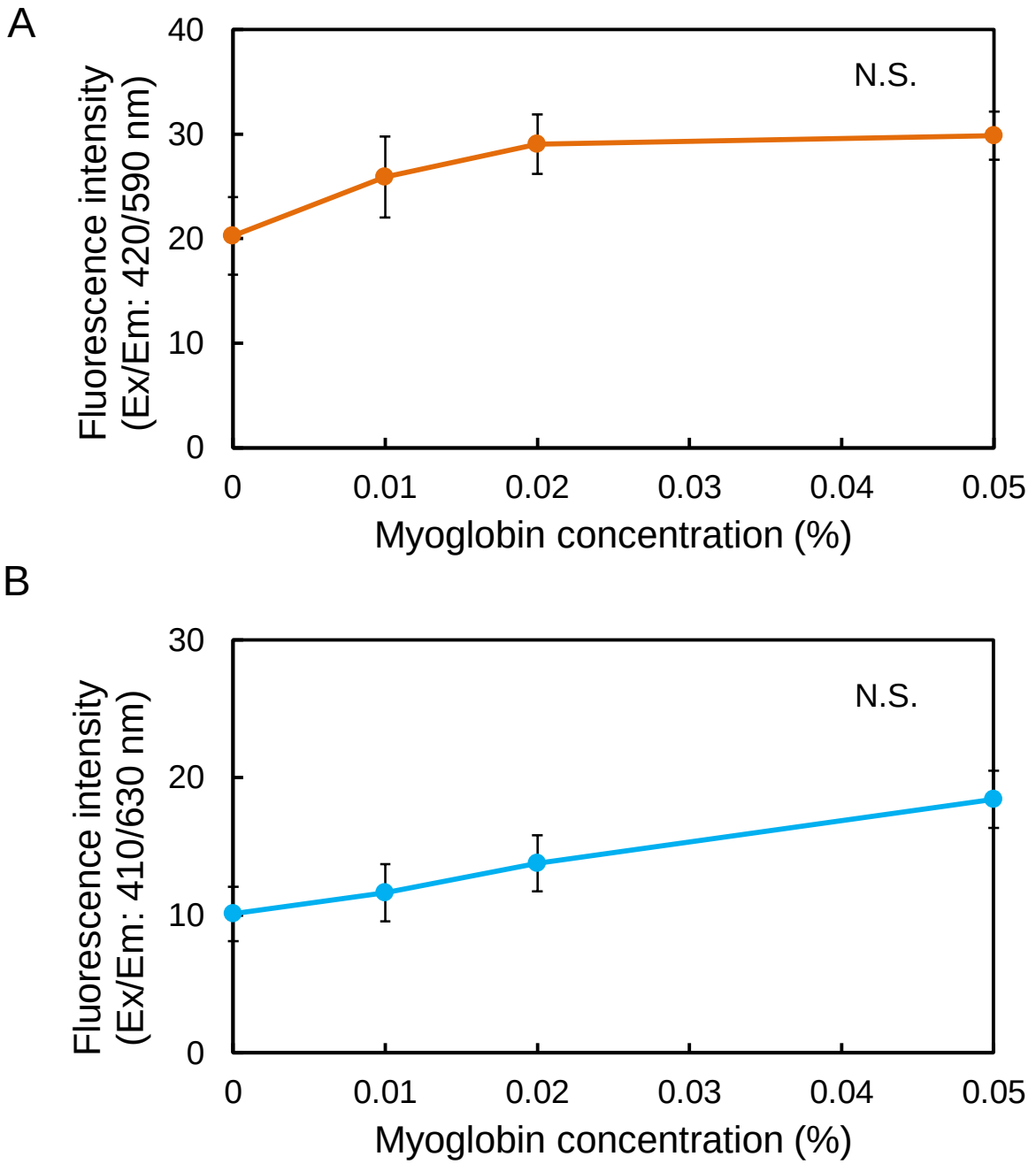


Fig. 34. Effect of exogenous desalted myoglobin instead of >10 kDa soluble fraction on ZnPP (A) and PPIX formation in fractionated LD muscle at pH 5.5.

Desalted oxymyoglobin was added to the LD insoluble and <10 kDa soluble fractions in different concentrations. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of means (n = 3). N.S.: Non-significant.

3.2.5. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by gel filtration chromatography

Similarly to section 3.1.9., the LD water-soluble protein was separated by a gel filtration chromatography and applied to ZnPP and PPIX formation model experiment systems. When the separated fractions were incubated with the insoluble and <10 kDa soluble fractions individually, the higher ZnPP and PPIX formation were observed in earlier eluted fractions than the myoglobin-eluted fractions (Fig. 35). Subsequently, 10-30 kDa water-soluble fraction was also applied to gel filtration chromatography. When the separated 10-30 kDa fraction were incubated with the insoluble and <10 kDa soluble fractions, higher ZnPP and PPIX were formed in earlier eluted fractions compared with the myoglobin-eluted fractions (Fig. 36). This result also showed that ZnPP (Fig. 36A) and PPIX (Fig. 36B) were formed in the absence of myoglobin. Therefore, the present result suggested that higher molecular weight protein compared with myoglobin in the 10-30 kDa water-soluble fraction contributed to ZnPP and PPIX formation at pH 5.5.

3.2.6. Effect of ammonium sulfate fractionation on ZnPP and PPIX-forming ability of LD muscle water-soluble protein(s)

To clarify the protein contributed to ZnPP and PPIX formation at pH 5.5, the LD water-soluble protein was fractionated with 25, 40, 50, 75 and 100% saturation of ammonium sulfate (NH_4SO_4). The supernatant and the diluted precipitate were incubated with the insoluble and <10 kDa soluble fractions in single or mixed groups. The result showed that ZnPP (Fig. 37A) and PPIX (Fig. 37B) formation were tended to be decreased with the increase of NH_4SO_4. Moreover, the supernatant and precipitate mixed group also showed the lower formation of ZnPP as well as PPIX compared with the control. After protein precipitation by NH_4SO_4, the protein might be lost their

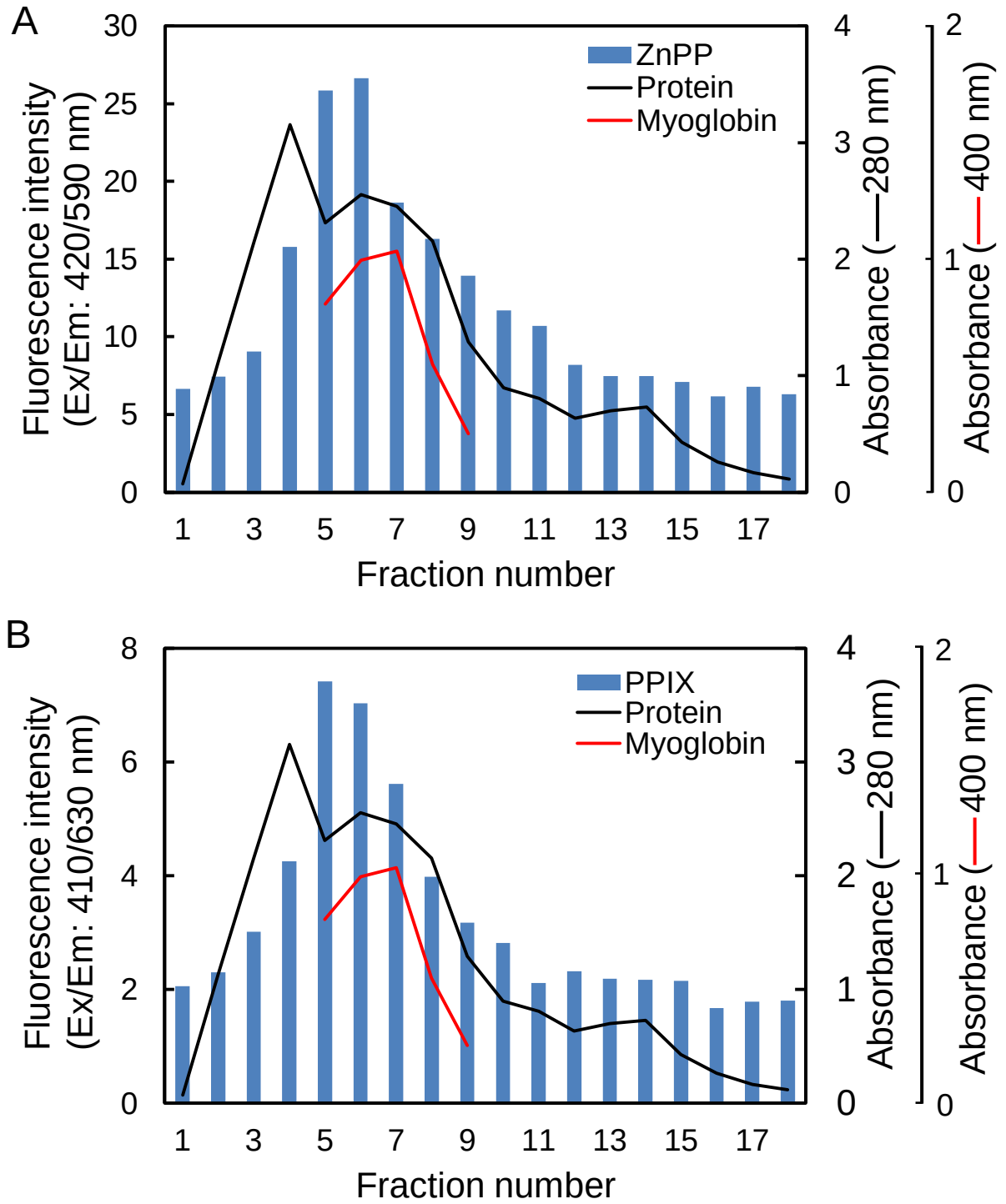


Fig. 35. ZnPP (A) and PPIX (B) forming ability of the gel filtrated fractions of IS water-soluble fractions at pH 5.5.

Concentrated LD water-soluble fraction was applied to the gel filtration chromatography (Toyopearl HW 50) and the eluted fractions were collected. The absorbance of protein and myoglobin were measured. Each eluted fraction was incubated with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured.

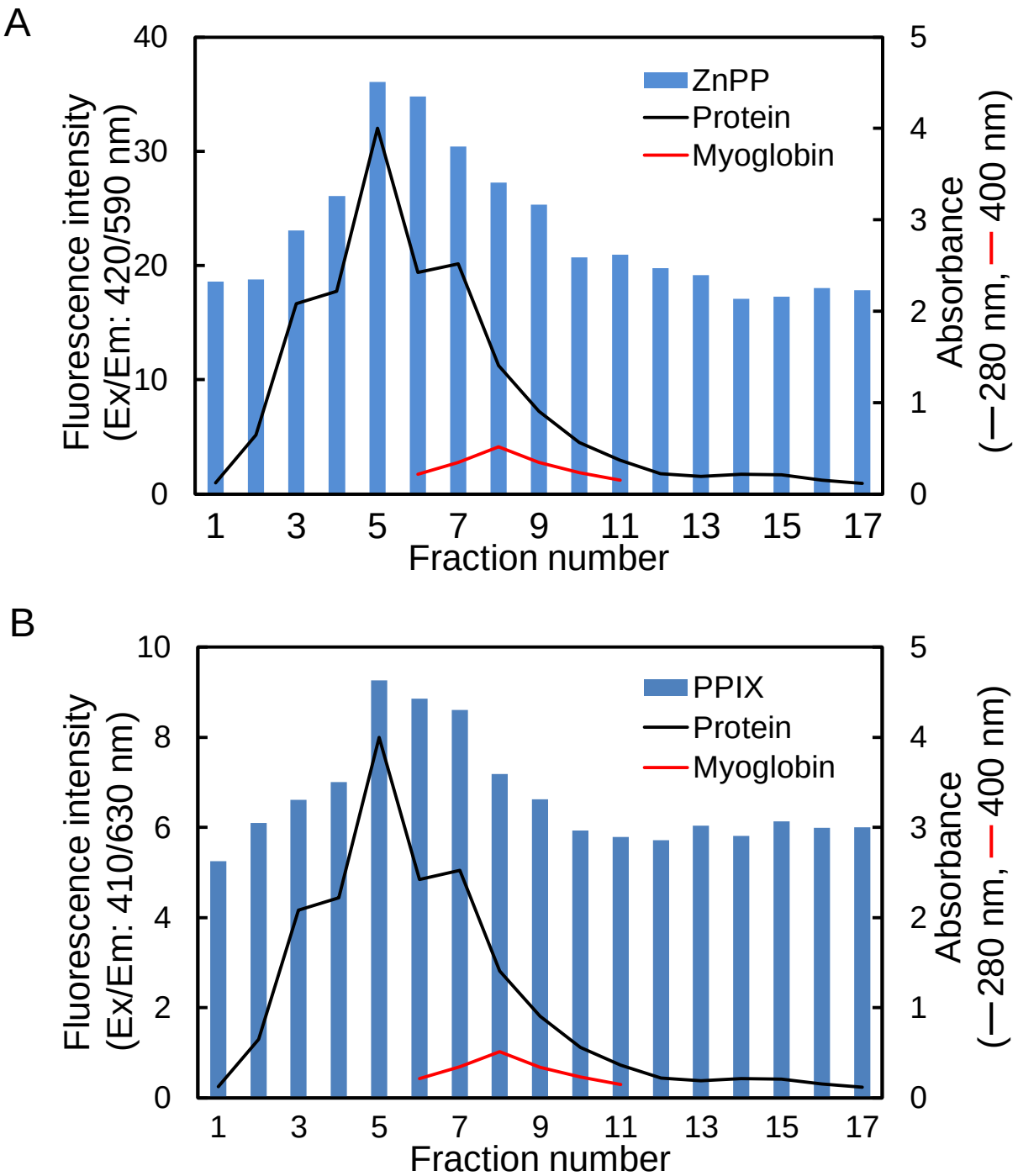


Fig. 36. ZnPP (A) and PPIX (B) forming ability of gel filtrated fractions of IS 10-30 kDa soluble fractions.

Concentrated LD muscle 10-30 kDa soluble fraction was applied to the gel filtration chromatography and eluted fractions were collected. The absorbance of protein and myoglobin were measured. Each eluted fraction was incubated with the insoluble and <10 kDa soluble fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured.

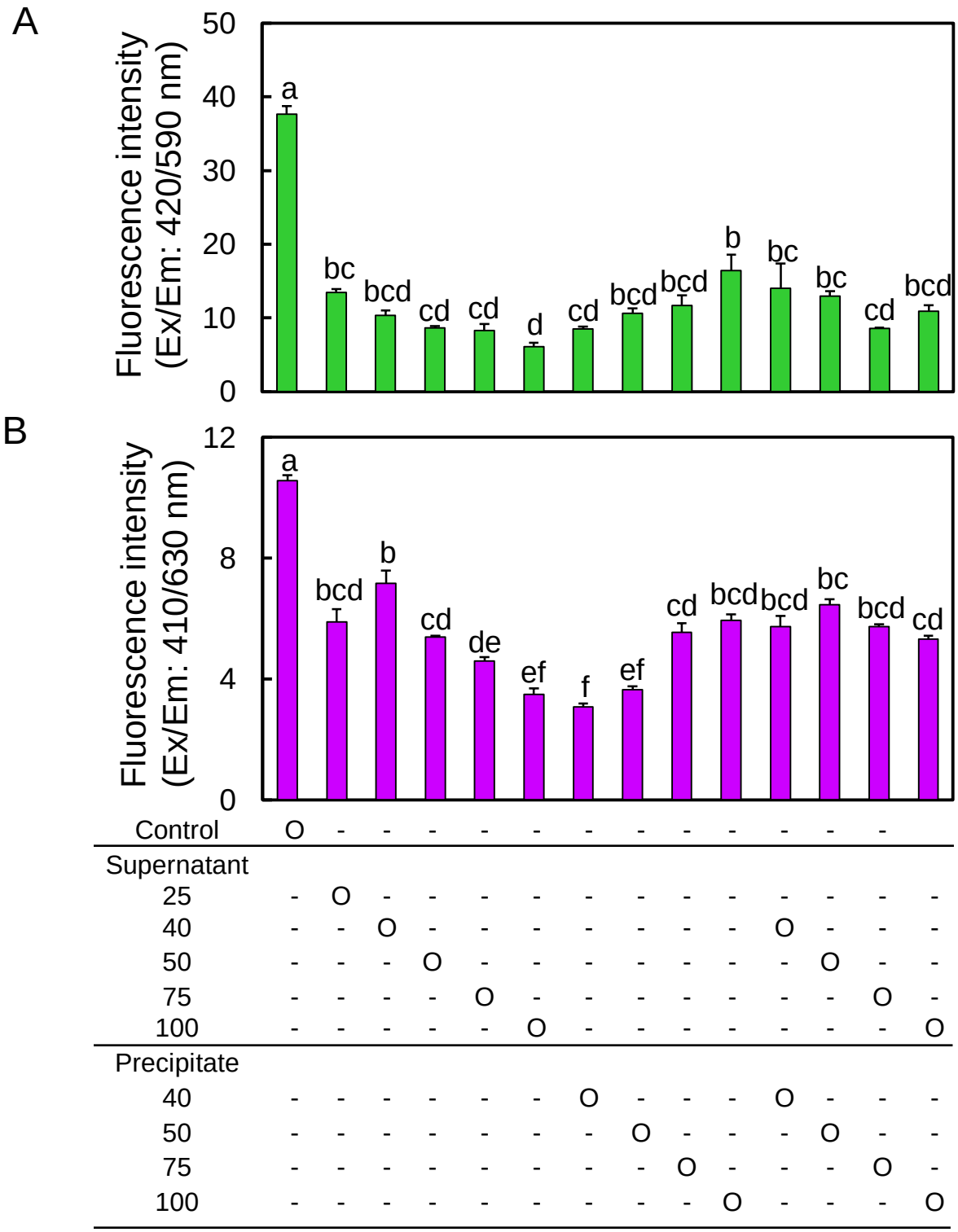


Fig. 37. Effect of ammonium sulfate fractionation on ZnPP (A) and PPIX (B) forming ability of the water-soluble protein at pH 5.5.

LD muscle water-soluble fraction was saturated with NH_4SO_4 concentration of 25, 40, 50, 75 and 100% and then separated into the supernatant and precipitate. The supernatant and precipitate were mixed with the insoluble and <10 kDa fractions. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically in the dark for 5 days at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of means (n = 3). S: Supernatant and P: Precipitate. abcdef: Values bearing different letters differ significantly ($P < 0.05$).

activity to work on ZnPP and PPIX formation model experiment systems, thus the formation of ZnPP and PPIX were might be suppressed.

3.2.7. ZnPP and PPIX-forming ability of the water-soluble protein(s) separated by cation exchange chromatography

To explore the contributing protein to ZnPP and PPIX formation at pH 5.5, next the LD water-soluble protein was separated by cation exchange chromatography (CIEX). When the fraction separated according to NaCl concentration was incubated with the insoluble and <10 kDa fractions, ZnPP (Fig. 38A) and PPIX (Fig. 38B) formation were significantly lower compared with control. Next, the separated fractions were incubated in single or mixed groups with the insoluble and <10 kDa soluble fractions. Myoglobin was not adsorbed under the condition and existed in 0 M fraction. The results showed that the amount of ZnPP (Fig. 39A) and PPIX (Fig. 39B) formed were significantly lower in any single and mixed groups than that in control. Therefore, the contributing protein might adsorb strongly with the used buffer pH or the elution with NaCl might inhibit the formation of ZnPP and PPIX at pH 5.5.

Next, the contributing possible protein in the water-soluble fraction was tried to elute with higher pH (up to 8.0) buffers. The water-soluble fraction was applied to a CIEX column equilibrated with buffer of each pH and the washing and collection with the same pH buffers (without NaCl) were performed. When the collected sample was incubated with the insoluble and <10 kDa soluble fractions, ZnPP formation was suppressed up to pH 7.5 but was rescued at pH 8.0 like control (Fig. 40A). However, the formation of PPIX was suppressed up to pH 8.0 (Fig. 40B). Although the kind of separated proteins was increased with the increase of pH as shown in SDS-PAGE image, the obvious difference between 7.5 and 8.0 was not observed (Fig. 41). Thus, it

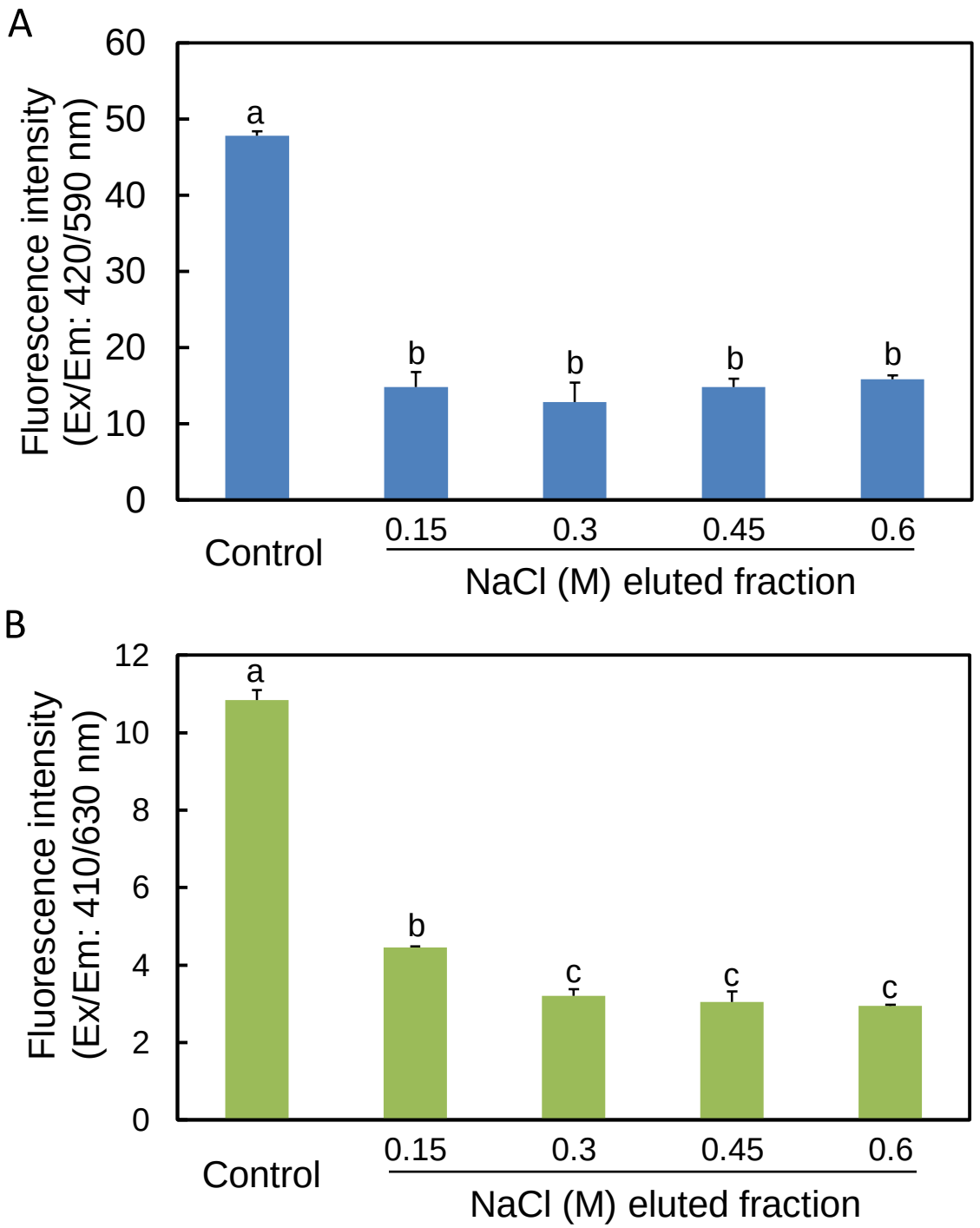


Fig. 38. ZnPP (A) and PPIX (B) forming ability at pH 5.5 of fractions eluted with different NaCl concentrations from CIEX column.

LD muscle water-soluble fraction was subjected to cation exchange gel column (CM-Toyopearl 650M). Then the sample was first washed with 10 mM citrate buffer (pH 5.5), subsequently stepwise eluted with 0.15, 0.3, 0.45 and 0.6 M NaCl. The eluted fractions were collected and mixed with the insoluble and <10 kDa soluble fractions. After addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically in dark for 5 days at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). abc: Values bearing different letters differ significantly ($P < 0.05$).

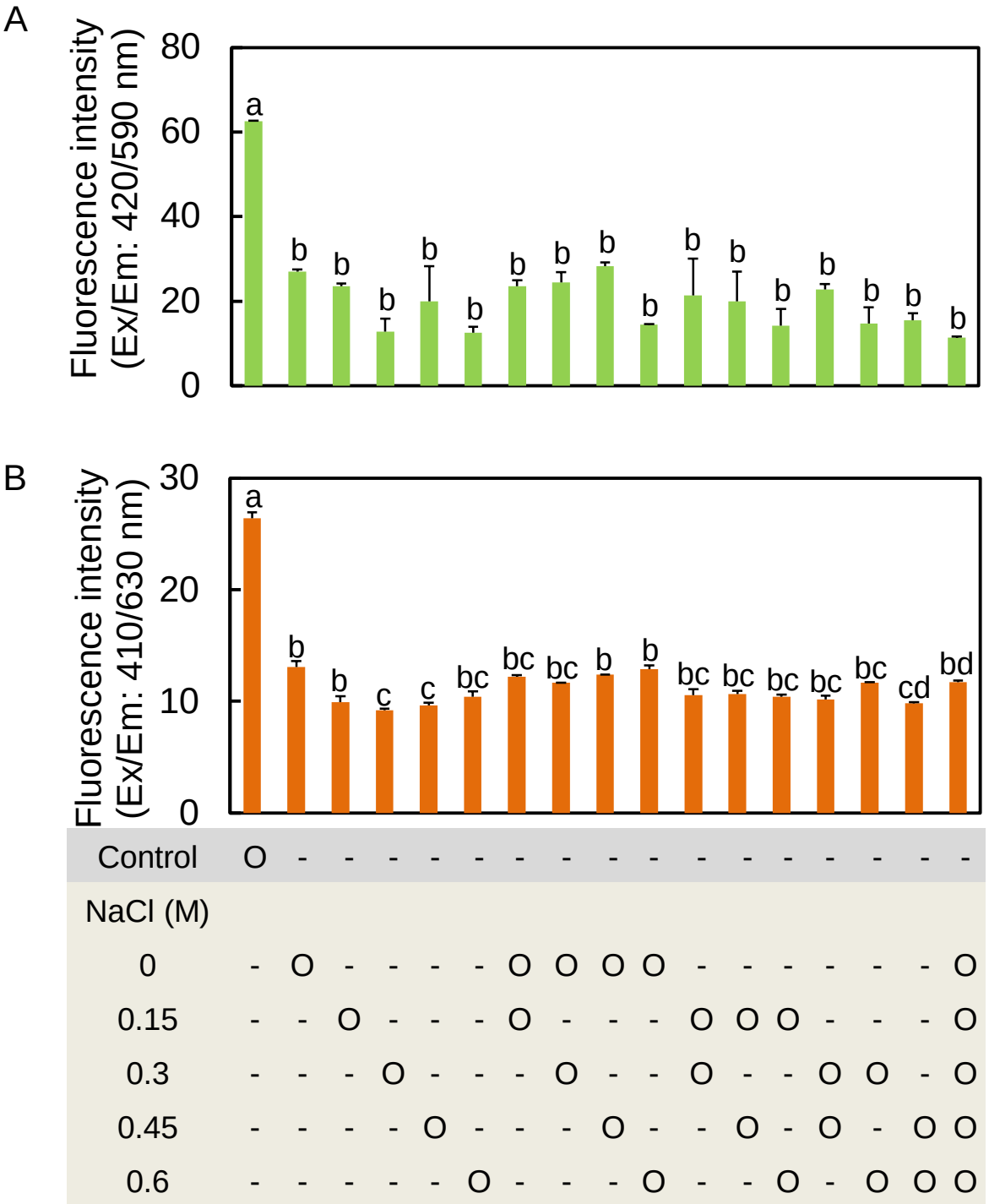


Fig. 39. Effect of combination of fractions eluted with different NaCl concentrations from CIEX column at pH 5.5.

LD muscle water-soluble fraction was first washed with 10 mM citrate buffer (pH 5.5), subsequently stepwise eluted with 0.15, 0.3, 0.45 and 0.6 M NaCl. The fraction was concentrated and mixed with the insoluble and <10 kDa soluble fractions in single and mixed groups. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). ab: values bearing different letters differ significantly ($P < 0.01$).

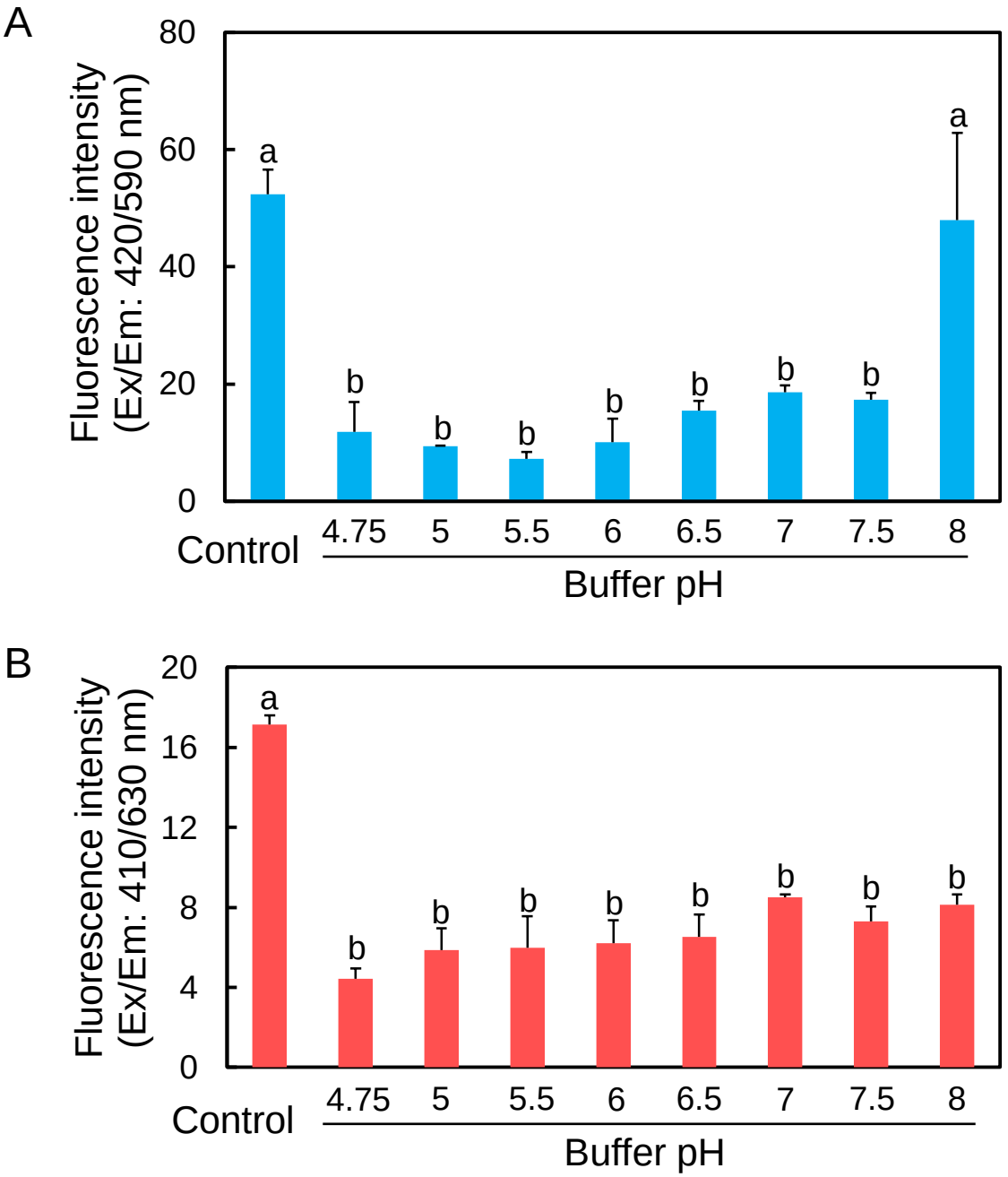


Fig. 40. ZnPP (A) and PPIX (B)-forming ability at pH 5.5 of the unadsorbed fractions to a CIEX column at the various pH.

LD muscle water-soluble fraction was applied to a CIEX column equilibrated with 10 mM citrate buffer (pH 4.75-8.0). The unadsorbed fraction was collected and concentrated. Then, the concentrated fractions were mixed with the insoluble and <10 kDa soluble fractions separately. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 2). ab: values bearing different letters differ significantly ($P < 0.01$).

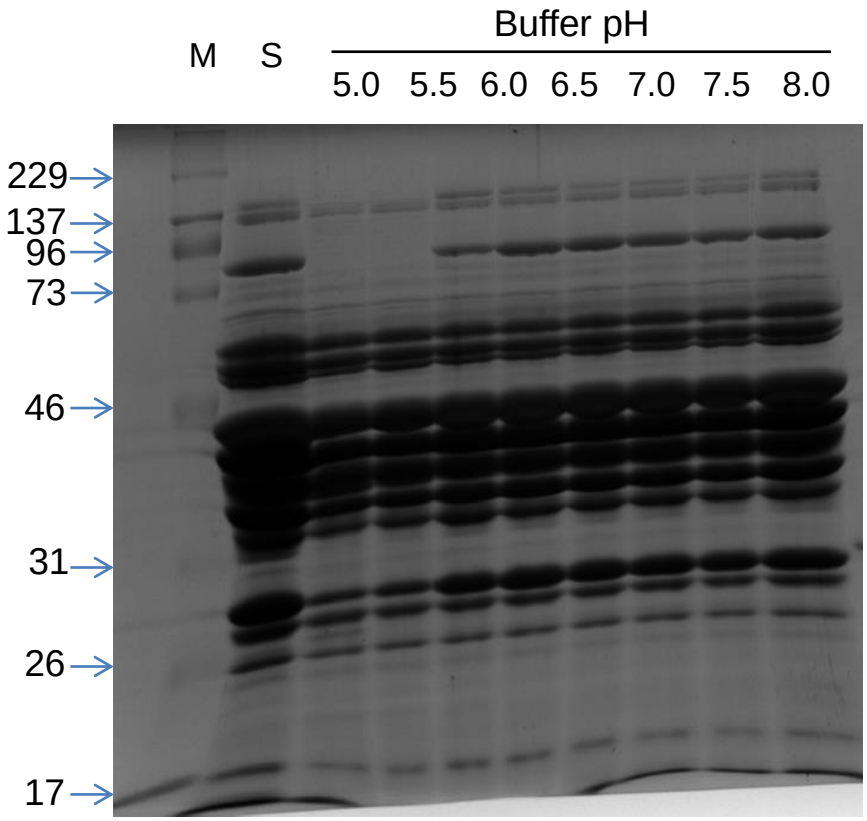


Fig. 41. SDS-PAGE image of the unadsorbed fractions to a CIEX column at various pH.

SDS-PAGE of each unabsorbed fraction (Fig. 40) was performed using 4.5% acrylamide for the stacking gel and 15% acrylamide for the separating gel. The gel was stained with CBB. M: Marker, S: Soluble fraction of LD muscle homogenate.

was revealed that the contributing possible protein in ZnPP formation at pH 5.5 was not adsorbed with a CIEX column at pH 8.0.

Then, the separation of the contributing protein that was not adsorbed with pH 8.0 buffer was tried. The water-soluble fraction was first applied to a CIEX column (pH 8.0) and washed with pH 8.0 (0 M NaCl) buffer. Next, the washed fraction was again applied to a CIEX column (pH 7.5) and the column was washed with pH 7.5 (0 M NaCl) buffer and the unadsorbed fraction was collected. Subsequently the column was eluted with pH 7.5 (0.6 M NaCl) buffer. The SDS-PAGE image of the three separated fractions was shown in Fig. 42. The band pattern of proteins that was not adsorbed with pH 8.0 (0 M NaCl) buffer was the almost same as that of protein washed with pH 7.5 (0 M NaCl) buffer, whereas the two 42 and 60 kDa bands were observed in the fraction eluted with pH 7.5 (0.6 M NaCl) buffer. Next, ZnPP and PPIX-forming ability of these separated fractions were examined by ZnPP and PPIX formation model experiment systems. When the pH 7.5 (0 M NaCl) buffer-washed and pH 7.5 (0.6 M NaCl) buffer-eluted fractions were incubated separately with the insoluble and <10 kDa soluble fractions, the amount of ZnPP formed in pH 7.5 (0.6 M NaCl) buffer-eluted fraction group was significantly higher than that in the pH 7.5 (0 M NaCl) buffer-washed fraction (Fig. 43A). However, the amount of PPIX formed was not significant between the two fractions (Fig. 43B). Thus, it is possible that water-soluble proteins having a molecular weight of 42 and/or 60 kDa contribute to the formation of ZnPP but does not contribute to the formation of PPIX at pH 5.5.

3.2.8. Amino acid sequencing

Next, in order to identify the 42 and 60 kDa soluble proteins, the amino acid sequencing of Edman degradation was performed. In case of 60 kDa proteins only four

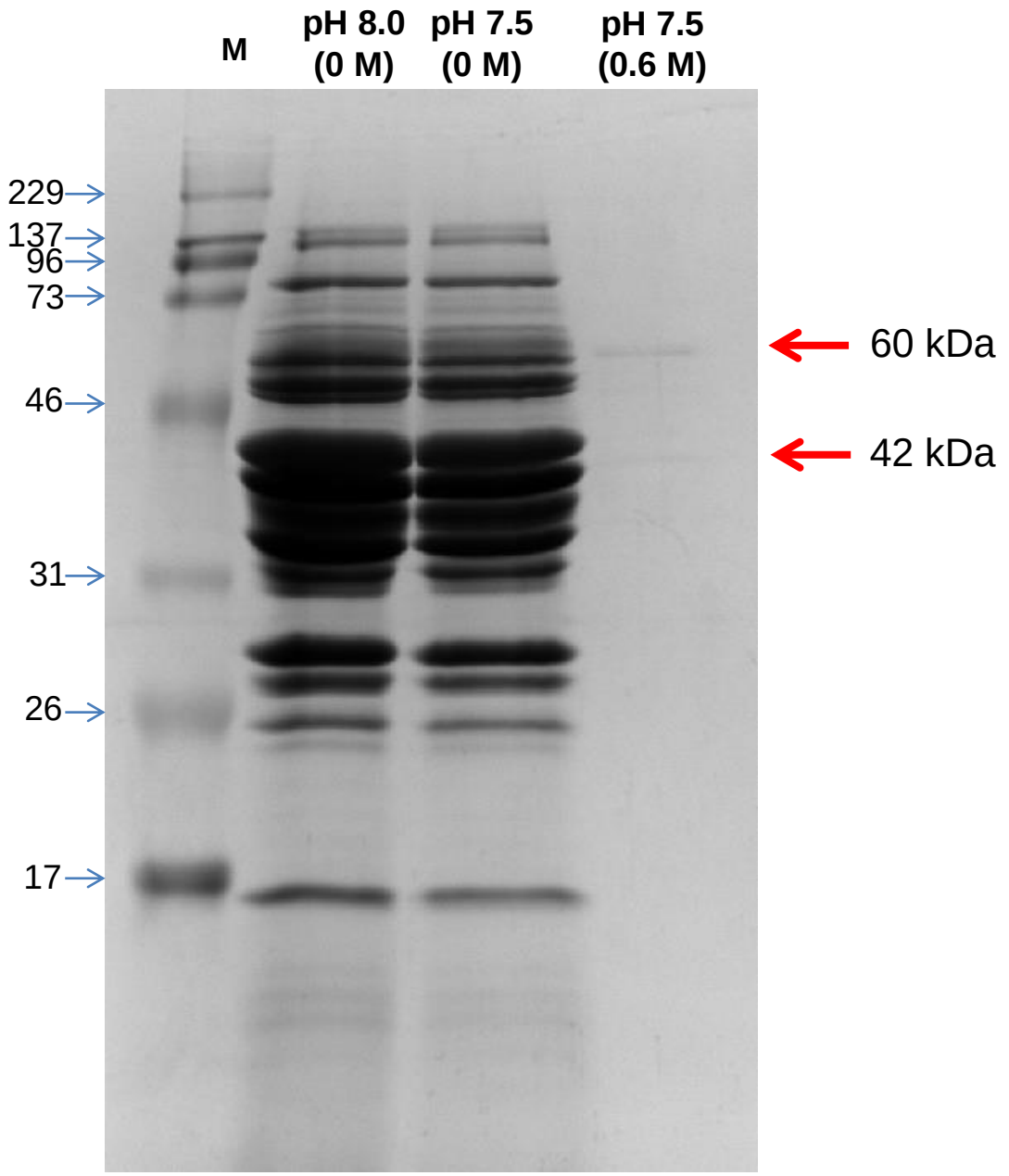


Fig. 42. Separation of ZnPP-forming possible contributors in the soluble fractions by CIEX column under high pH condition.

LD muscle water-soluble fraction was subjected to CIEX column (pH 8.0). Then the sample was washed with 10 mM citrate buffer of pH 8.0 (0 M NaCl). Then the washed fraction was again applied to the gel column (pH 7.5) and the column was washed with buffer pH 7.5 (0 M NaCl) and subsequently eluted with buffer pH 7.5 (0.6 M NaCl). The non-adsorbed fractions were collected and concentrated. The pH 7.5 (0.6 M NaCl) buffer fraction was dialyzed. The SDS-PAGE of collected fraction was performed using 4.5% acrylamide for the stacking gel and 15% acrylamide for the separating gel and stained with CBB. M: Marker.

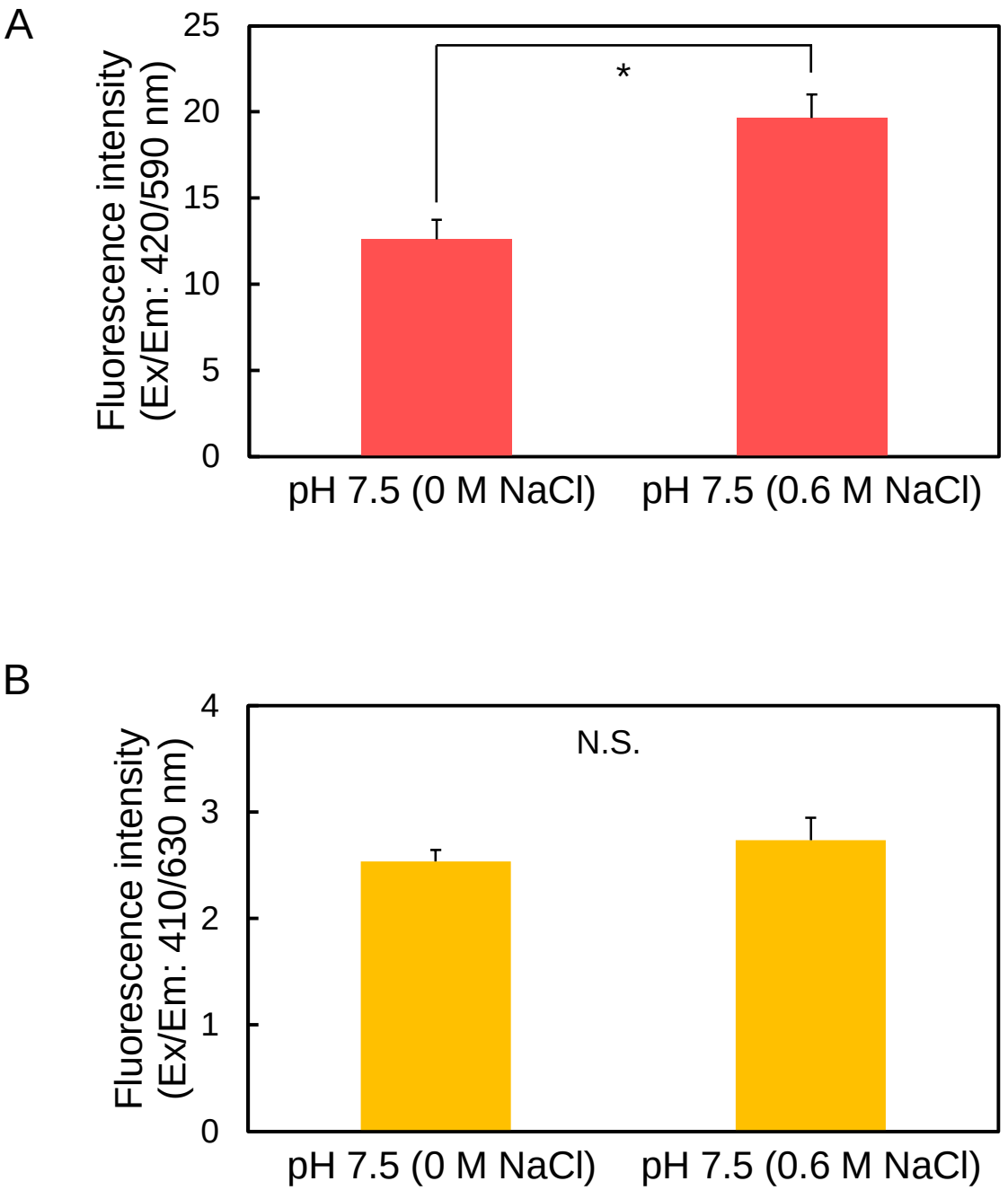


Fig. 43. ZnPP (A) and PPIX (B) forming ability of the separated protein by CIEX in LD muscle.

After collection of each washed and eluted fraction (Fig. 42), these were mixed with the insoluble and <10 kDa soluble fractions separately. After the addition of antibiotics and EDTA (for PPIX formation), the solution was incubated anaerobically for 5 days in the dark at 25°C. ZnPP and PPIX were extracted and the fluorescence intensity was measured. Bars represent the standard error of the means (n = 3). *Significant difference at $P < 0.05$. N.S.: Non-significant.

residue sequence (EMPF) was decided due to the insufficient amount of sample or chemical modification of N-terminal region in Edman degradation method. Although the sequence was search with a data base (BLASTP), many porcine proteins were hit and it could not be identified. On the other hand, the amino acid sequence of 42 kDa protein was not decided due to the insufficient amount of sample or chemical modification of N-terminal region in Edman degradation method. However, the identification of 42 and 60 kDa components will be helpful to make the relationship of this protein and ZnPP formation at pH 5.5.

4. Discussion

The Italian traditional dry-cured ham (Parma ham) shows a stable bright red color derived from ZnPP (Wakamatsu et al., 2004a). In order to elucidate the formation mechanism of ZnPP in pork, this research has studied about ZnPP formation mechanism in the optimum pH at 4.75 and 5.5.

4.1. Mechanism of ZnPP formation at pH 4.75

4.1.1. ZnPP-forming route

In our laboratory, a new optimum pH for ZnPP formation was observed at pH 4.75 (Honma, 2014). But, from where ZnPP is derived at pH 4.75? The chelation of zinc by a chelator EDTA (Adamsen et al., 2006a) was predicted to inhibit the formation of ZnPP at pH 4.75 and results in the accumulation of PPIX in model solution (Wakamatsu et al., 2007; Becker et el., 2012). It was also found that ZnPP in a model solution was the insertion of Zn into PPIX, which was formed independently (Wakamatsu et al., 2007). However, the investigations of the effects of various factors on ZnPP and PPIX formation revealed that ZnPP formation (Fig. 7) was almost the same as that on PPIX formation at pH 4.75 (Fig. 8). This result is consistent with the result at pH 5.5 (Wakamatsu et al., 2007). Therefore, ZnPP was suggested to derive from PPIX in the mechanism of ZnPP formation at pH 4.75.

The next question is from where PPIX is derived? The earlier studies suggested that the bright red color in Parma ham is caused by ZnPP in which the iron in the heme of myoglobin has been replaced by zinc (Wakamatsu et al., 2004a) since the structures of heme and ZnPP are similar. At pH 5.5, PPIX is not from heme in myoglobin

(Wakamatsu et al., 2007). However, this study indicated that the exogenous myoglobin promoted to form PPIX at pH 4.75 (Fig. 17B). Moreover, fractions that was eluted myoglobin by gel filtration increased the formation of PPIX (Fig. 23B and 24B). Ishikawa et al. (2007) suggested that mitochondria have the ability to form PPIX from oxymyoglobin and are directly related to the release of Fe^{2+} from porphyrin ring in myoglobin. Ishikawa et al. (2006) also reported that ZnPP was formed from myoglobin in pork loin extract. Therefore, PPIX at pH 4.75 was suggested to be derived from heme in myoglobin in ZnPP formation mechanism at pH 4.75. Hence, it follows that ZnPP is formed from heme of myoglobin via PPIX.

4.1.2. Mechanism of PPIX formation from heme derived myoglobin

Afterward, in order to clarify the mechanism of PPIX formation from heme derived from myoglobin, the IS muscle homogenate was fractionized. When IS muscle homogenate was separated into three fractions i.e. the insoluble fraction, the >10 kDa soluble fraction, and the <10 kDa soluble fraction, the formation of PPIX was inhibited in the absence of one of the three fractions (Fig. 10). In the previous report (Shirashi, 2010), the effect of fractionation on PPIX formation has not been checked yet but the high molecular and low molecular water-soluble fraction and insoluble fractions of *longissimus* muscle homogenate are necessary for the formation of ZnPP at pH 5.5. This study suggested that one or more components in each fraction (the insoluble fraction, the >10 kDa soluble fraction and <10 kDa soluble fraction) contributes to form PPIX at pH 4.75.

The insoluble fraction used in this study contains mainly myofibril, connective tissue and organelle such as nucleus, mitochondria. FECH enzymes are located in the inner mitochondrial membrane. Because FECH that is considered to be involved in the

formation of ZnPP (Becker et al., 2012; Benedini et al., 2008; Parolari et al., 2009; Wakamatsu et al., 2007) is the terminal enzyme of the heme-biosynthetic pathway in mitochondria, it is likely to be present in the insoluble fraction of pork homogenate. FECH also involved in the iron removal reaction from heme to form PPIX (Chau et al., 2011). In the fractionation of this study, FECH would be exist in the insoluble fraction which is essential for the formation of PPIX at pH 4.75 (Fig. 10). FECH was observed in mitochondria of Parma ham and its localization almost coincided with that of ZnPP (Shimoda, 2014). Hence FECH from the insoluble fraction is supported to be one of the contributors to PPIX formation.

Regarding the >10 kDa soluble fraction, it was revealed that myoglobin from the >10 soluble fractions and another water-soluble protein contribute to the formation of PPIX at pH 4.75. Water-soluble fraction of pork homogenate contains many sarcoplasmic proteins and most of the sarcoplasmic proteins are present in the >10 kDa soluble fraction. Myoglobin is a sarcoplasmic heme protein primarily responsible for the color of meat (Livingston & Brown, 1981) and according to its molecular weight (17 kDa), would be in the >10 kDa soluble fraction. In this study the addition of exogenous myoglobin and gel filtrated-fraction with myoglobin promoted to form PPIX. Grossi et al. (2014) reported that proteolytic degradation of myoglobin and precipitation of inorganic iron (III) in the meat matrix are essential for the metal exchange to form ZnPP and non-heme iron (III) species. Wakamatsu et al. (2019) also suggested that ZnPP formation at pH 4.75 is derived from myoglobin. Therefore, myoglobin was suggested to be a contributor in this fraction. On the other hand, the fraction eluted together with myoglobin in CIEX column did not promoted to form PPIX (Fig. 20B). It is possible that the protein with similar molecular weight of myoglobin contributed to the

formation of PPIX. In order to verify the existence of the contributor, effect of the combination of fractions eluted with CIEX column on PPIX formation was checked (Fig. 19B). But PPIX was not formed in any combinations. The sarcoplasmic proteins are a mixture of several hundred molecular species the complexity of which has been shown by modern proteomic techniques such as two-dimensional electrophoresis (Bendixen, 2005). It is possible that another candidate was lost the ability to form PPIX by the elution from CIEX column or was not eluted due to the strong adsorption with CIEX column. Regarding the other contributors, further experiments are needed. Therefore, in the >10 kDa soluble fraction, myoglobin and unknown water-soluble protein play a role to form PPIX at pH 4.75.

It was revealed that the heat-stable component present in the <10 kDa soluble fraction was essential for the formation of PPIX (Fig. 10). There are many low molecular weight components such as amino acids, vitamin $B_{1,}$ pantothenic acid, zinc ion and iron ion that are present in the <10 kDa soluble fractions of pork homogenate. Among them, vitamin B_1 and pantothenic acid are heat-sensitive. Therefore, it is suggested that such heat-sensitive compounds do not contribute to PPIX formation at pH 4.75. Accordingly, if only Zn^{2+} ion in the <10 kDa soluble fraction is the contributor, mixing of the insoluble and >10 kDa soluble fractions with EDTA would increase the PPIX formation. Otherwise, if other heat-stable contributor other than Zn^{2+} exists in the <10 kDa soluble fraction, the obtained result also might be reasonable. Further investigations are needed to find out the identity of the <10 kDa soluble component contributing to PPIX formation at pH 4.75.

It was suggested that ZnPP is formed from heme in myoglobin (Wakamatsu et al., 2004b). However, is heme in myoglobin deironized directly? Heme is located in the

globin pocket of myoglobin and is not exposed out of myoglobin. Therefore, it would be difficult for FECH to catalyze directly from the outside of intact myoglobin. Degradation of globin or release of heme from myoglobin might be needed to the formation of PPIX from heme. Accordingly, Honma (2016) reported that significant degradation of myoglobin was observed after 5 and 10 days of incubation at pH 4.75. It was also reported that denaturation of myoglobin has been found to occur in the pH region 4.0-5.0, and this denaturation leads to the detachment of the heme group from myoglobin (Steinhardt et al., 1963; Allis & Steinhart, 1970). In present study, degradation of globin or release of heme from myoglobin was not elucidated. This study hypothesized that the heme is released or exposed from globin pocket of myoglobin at pH 4.75 and subsequently PPIX is formed via iron-removal reaction from heme.

Therefore, it is speculated that degradation of myoglobin or release of heme from myoglobin could be occurred due to low pH and FECH from insoluble fraction catalyzes the iron-removal reaction from heme and PPIX is formed. In addition, heat-stable components except zinc ion also contributed to the formation of PPIX at pH 4.75.

4.1.3. Mechanism of ZnPP formation from PPIX

It was reported that ZnPP in a model solution was the insertion of Zn into PPIX (Wakamatsu et al., 2007). The formation of ZnPP was inhibited in the absence of one of the three pork homogenate fractions. But it is not shown if all fractions of pork homogenate are essential to insert of Zn into PPIX. In this experiment system, the essential components for PPIX formation are the essential components for ZnPP formation but the opposite cannot be indicated either. Moreover, the essential contributors in the formation of ZnPP from PPIX cannot be indicated. Therefore, the above-mentioned components derived from the three fractions might contribute to form

ZnPP from PPIX. The addition of $NaNO_2$ and N-MMP inhibited ZnPP and PPIX formation indicating that FECH is deeply involved in the formation of ZnPP and PPIX at pH 4.75. Mammalian ferrochelatase contains a [2Fe–2S] cluster (Ferreira et al., 1999; Dailey et al., 2000), and the cluster is labile and sensitive to destruction by NO (Sellers et al., 1996). N-MMP has been established as potent inhibitors of FECH (Tephly et al., 1979), and inhibited the formation of ZnPP at low concentrations in meat homogenates (Becker et al., 2012). Indeed, FECH catalyzes the insertion of divalent metal into PPIX. On the other hand, where is Zn derived from? Zinc in meat exist as ion or Zn-binding protein and so on. It is assumed that Zn exists in the <10 kDa soluble fraction as free ion and exists in the insoluble and >10 kDa soluble fractions as Zn-binding protein. Not only ZnPP but also PPIX was not formed in the absence of the <10 kDa soluble fraction. Adamsen et al. (2006a) was found a positive correlation between both Zn content and Fe content and the logarithmic transformed ZnPP content. Benedini et al. (2008) also reported that a mixture of the Zn^{2+} and PPIX has moreover been shown only to form ZnPP in the presence of meat extract. It was reported that zinc is present in muscles both in insoluble and soluble component (Hazell, 1982). On the other hand, Zn is the second easiest metal after copper for insertion into porphyrins and is easily inserted into porphyrin without the requirement of a catalyst (Becker et al., 2012). Therefore, based on the previous reports and these study findings, it is suggested that Zn ion either from the insoluble or soluble fraction contributed to the formation of ZnPP from the precursor PPIX at pH 4.75 with FECH or non-enzymatically. In addition, the heat-stable soluble component also might contribute to ZnPP formation from PPIX.

4.1.4. Proposed ZnPP formation mechanism at pH 4.75

Together with the results obtained, the mechanism by which ZnPP is formed in meat and meat products is proposed in Fig. 44. The globin in myoglobin is degraded or the heme molecule is released from myoglobin due to low pH 4.75. Subsequently, from the insoluble fraction FECH catalyzes the iron removal reaction from heme in association of the heat-stable <10 kDa soluble compounds (except zinc ion) and PPIX is formed. Then, the zinc ion is inserted into PPIX either from insoluble or >10 kDa soluble fraction and produced ZnPP by the catalysis of FECH or spontaneously. It is also suggested that the heat-stable <10 kDa soluble component also facilitates the formation of ZnPP from PPIX in accordance with FECH or non-enzymatically.

4.2. Mechanism of ZnPP formation at pH 5.5

4.2.1. ZnPP-forming route

It was reported that PPIX is a precursor of ZnPP formation at pH 5.5 (Wakamatsu et al., 2007). But, from where PPIX is derived? It was reported that PPIX is the final intermediate in the heme synthesis pathway in the living body. PPIX is formed by protoporphyrinogen oxidase and converted to heme by FECH, both are mitochondrial enzymes (Ajioka et al., 2006). In the previous study, myoglobin was not degraded after incubation at pH 5.5 (Honma, 2016). It was also suggested that PPIX is formed by the heme biosynthetic pathway (Shirashi, 2010). The amount of PPIX formed exogenously in model sample has been shown to increase significantly by adding coproporphyrinogen III, which serves as a substrate for PPIX in the heme biosynthetic pathway (Hayashi, 2009). Therefore, PPIX in model solution might be accumulated via

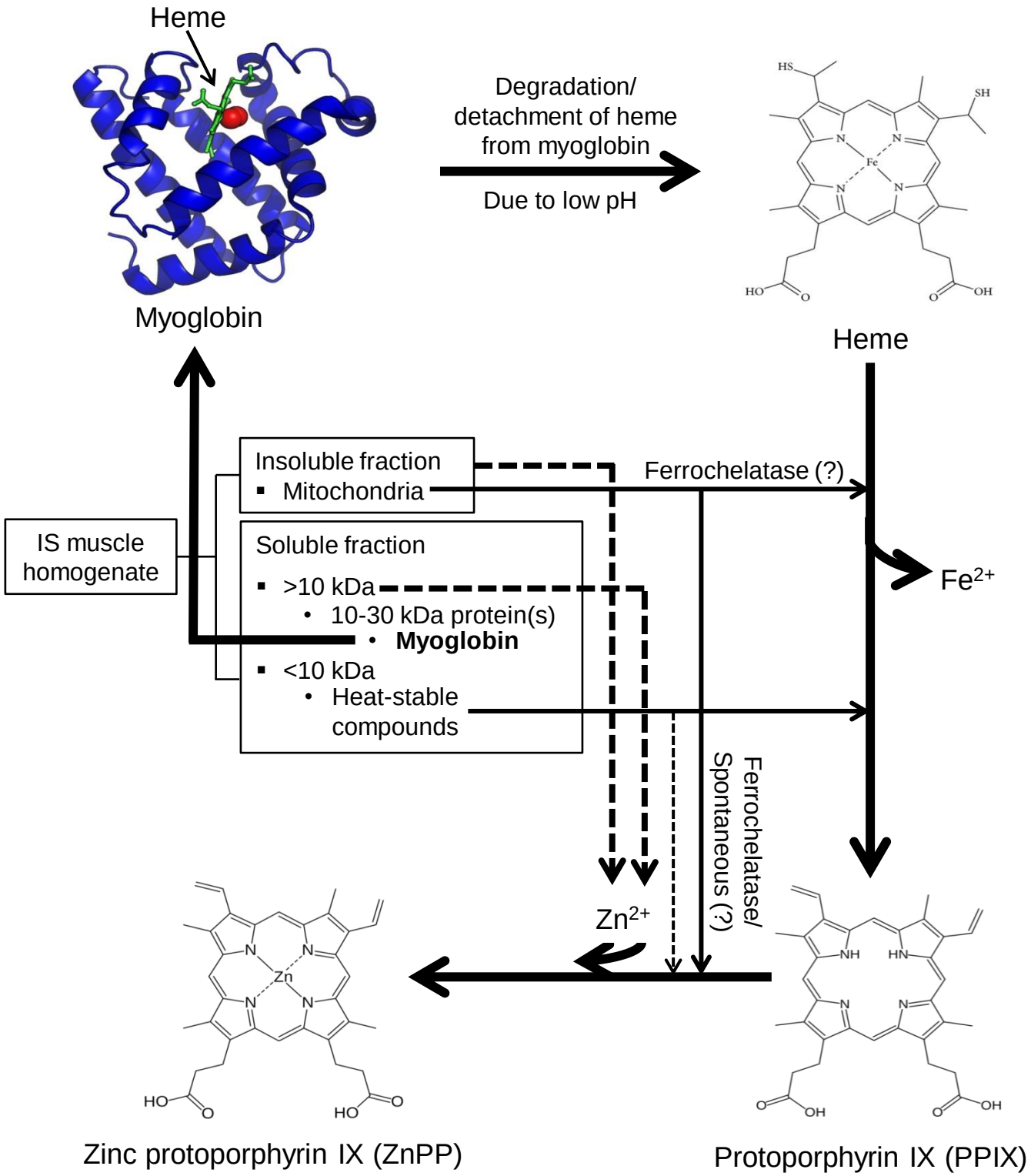


Fig. 44. Scheme 1: Proposed mechanism of ZnPP formation at pH 4.75 in pork.

Suggestion of origin

Speculation of origin

Suggestion of contribution

Speculation of contribution

a precursor(s) which is unknown or produced from the heme biosynthetic pathway but not from heme in myoglobin.

4.2.2. Mechanism of PPIX formation

In order to know the formation mechanism of PPIX, this study fractionized the LD muscle homogenate. When LD muscle homogenate was separated into three fractions i.e. the insoluble fraction, the >10 kDa soluble fraction, and the <10 kDa soluble fraction, the formation of PPIX was suppressed in the lack of one of those fractions. These results are similar to the results at pH 4.75 (Fig. 10) and the previous study (Shirashi, 2010). Thus, one or more components in each separated fraction are suggested to contribute to form PPIX at pH 5.5.

The insoluble fraction used in this study contains mitochondria as above-mentioned. FECH would be present inner mitochondrial membrane of the insoluble fraction. FECH was observed in the mitochondria of full matured-Parma ham (Shimoda, 2014). It was reported that FECH from porcine muscle mitochondria catalyzes the insertion reaction of zinc into PPIX to form ZnPP (Ishikawa et al., 2007; Chau et al., 2010; Parolari et al., 2009). This result suggested that FECH contributed to the formation of PPIX at pH 5.5.

Next, the contribution of water-soluble protein was observed for PPIX formation at pH 5.5. From the fractionation and ultrafiltration study, it was observed that the 10-30 kDa soluble fraction are important for PPIX formation at pH 5.5 (Fig. 28). However, the addition of exogenous myoglobin is not essential for the formation of PPIX (Fig. 34). Fractions having higher molecular weight protein than that of myoglobin promoted the formation of PPIX (Fig. 36). It was reported that exogenous myoglobin is not essential for ZnPP formation at pH 5.5 (Shirashi, 2010; Wakamatsu et al., 2007). Khozroughi et

al. (2017) reported that ZnPP is formed by a Fe^{2+}-Zn^{2+} substitution in myoglobin heme where accompanying myoglobin degradation is not necessarily obligatory and other proteins might have been involved in the formation of the ZnPP as well. Since there is little evidence that ZnPP is formed from myoglobin at pH 5.5, it is possible that another protein has a role to ZnPP formation. Therefore, water-soluble protein other than myoglobin contributed to PPIX formation at pH 5.5.

Heat-stable components in the <10 kDa soluble fraction was essential to form PPIX at pH 5.5. There are many low molecular heat-stable components including zinc ion present in the <10 kDa soluble fraction. In this experiment mixing of the insoluble and >10 kDa soluble fractions with EDTA did not increase PPIX formation similarly as at pH 4.75. Shirashi (2010) reported that ZnPP formation at pH 5.5 is promoted by the addition of zinc up to 4 mM and inhibited at higher concentrations. Therefore, divalent metal ion zinc in the <10 kDa soluble fraction might not contribute to PPIX formation at pH 5.5.

Therefore, it was suggested that FECH from the insoluble fraction, 10-30 kDa soluble protein other than myoglobin and heat-stable components except zinc ion also contribute to the formation of PPIX at pH 5.5.

4.2.3. Mechanism of ZnPP formation from PPIX

The final step in ZnPP formation is the insertion of Zn^{2+} to PPIX. The formation of ZnPP was suppressed in the absence of one of the three LD homogenate fractions (Fig. 26). But it was not shown if all fractions of LD homogenate are essential to insert of Zn into PPIX. Therefore, the above-mentioned components derived from the three fractions might contribute to form ZnPP from PPIX.

This study found that 42 and/or 60 kDa soluble components promoted to ZnPP formation at pH 5.5 (Fig. 43). But, in this study the 42 and/or 60 kDa molecular weight proteins did not identify by amino acid sequencing due to the insufficient amount of the sample chemical modification of N-terminal region in Edman degradation method. It was reported that molecular weight of the water-soluble ZnPP-binding protein in Parma ham estimated to be 38, 30 and 27 kDa; and the 27 kDa component was identified as long-chain-fatty-acid-CoA ligase I (Saito, 2016). However, 42 and 60 kDa protein has been identified as enolase (Luccia et al., 2005) and phosphoglycerate kinase (Gallego et al., 2016). If they could be identified, it would be a clue for the elucidation of ZnPP formation in Parma ham.

4.2.4. Proposed ZnPP formation mechanism at pH 5.5

Together with the obtained results and previous reports, the mechanism by which ZnPP is formed in meat and meat products at pH 5.5 is proposed in Fig. 45. PPIX is a precursor of ZnPP formation at pH 5.5 and PPIX is formed by the heme biosynthetic pathway. In fractionated LD muscle homogenate, FECH enzyme from the insoluble fraction and 10-30 kDa water-soluble protein other than myoglobin contributed to the formation of PPIX from heme biosynthetic pathway. Heat-stable components in the <10 kDa soluble fraction also contributed to PPIX formation process. Afterward, zinc ion either from the insoluble fraction or >10 kDa soluble fraction inserted into PPIX and produced ZnPP by the catalysis of FECH from the insoluble fraction or non-enzymatically. Moreover, 42 and/or 60 kDa components in the soluble fraction might play a role to insertion of Zn^{2+} into PPIX. It is also suggested that heat-stable

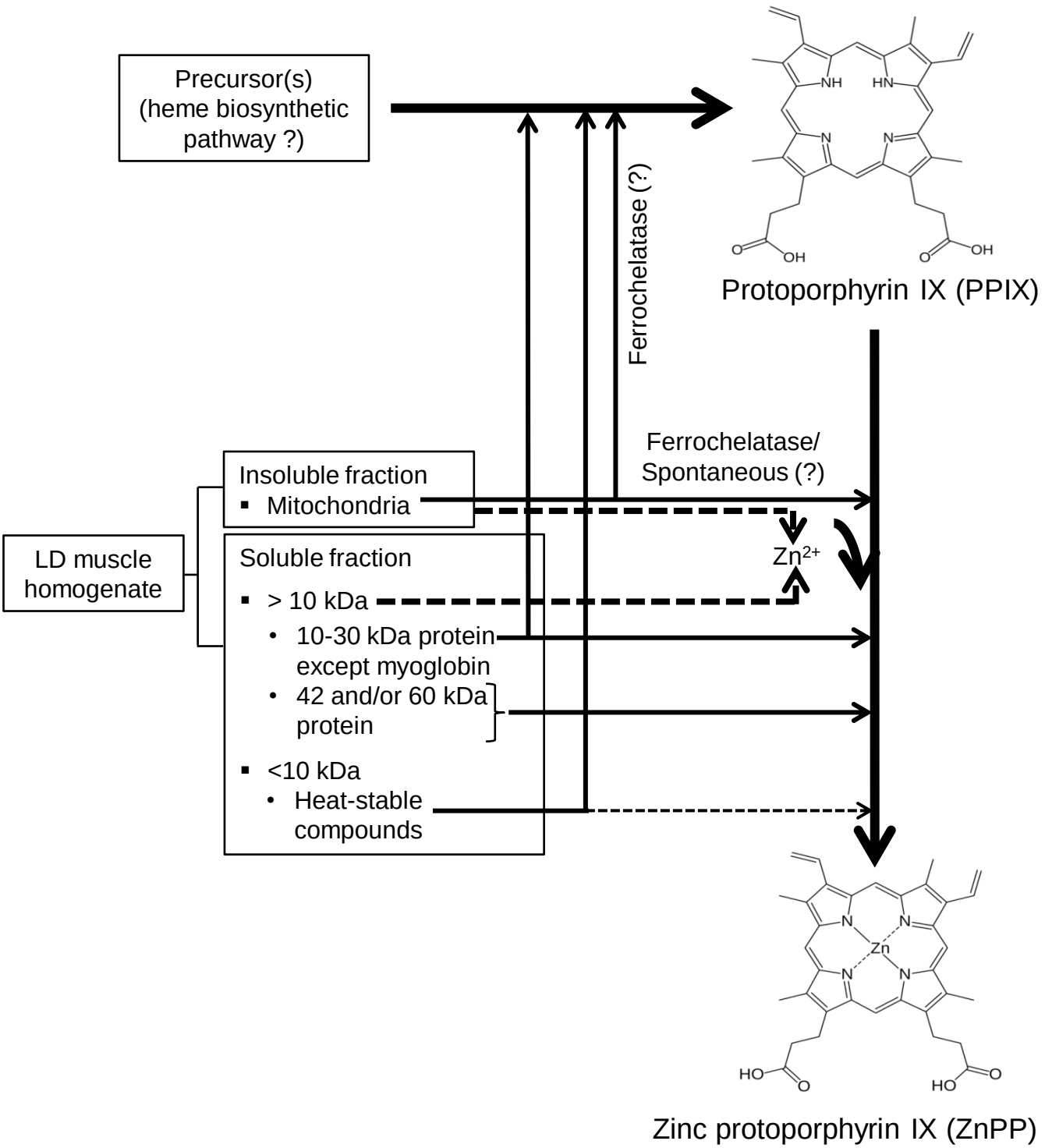


Fig. 45. Scheme 1: Proposed mechanism of ZnPP formation at pH 5.5 in pork.

Suggestion of origin

Speculation of origin

Suggestion of contribution

Speculation of contribution

components in the <10 kDa soluble fraction also contributed to the formation of ZnPP from PPIX at pH 5.5.

4.3. ZnPP formation mechanism in Parma ham

The current research result suggested that in terms of the incubation time, temperature, precursor level and the contribution of water-soluble proteins (Table 5), the formation mechanism of ZnPP is different between at pH 4.75 and 5.5 in pork. However, which mechanism is dominant in Parma ham? The pH of Parma ham is about 5.5-6.0. So, among the two optimum pH, is pH 5.5 mechanism dominant? However, since the ability of ZnPP formation at pH 4.75 is much higher than that at 5.5. The ZnPP mechanism at optimum pH 4.75 cannot be ignored in Parma ham. Except temperature, many factors influencing ZnPP/PPIX formation pattern are common such as oxygen, FECH inhibitor etc. and the mechanisms are thought to be complex. Several steps might be common among the formation mechanism at pH 4.75 and pH 5.5. Since the presence of FECH in mitochondria was also shown in dry-cured ham and suggested that FECH involved in ZnPP formation (Ozaki, 2017). ZnPP autofluorescence also differs depending on the muscle fibers, and ZnPP was abundantly present in the slow-twitch muscle fibers (Shimoda, 2012). Slow-twitch (type I) muscle fibers have more mitochondria and myoglobin than fast-twitch (type II) muscle fibers. Moreover, this study revealed that the precursors for ZnPP formation were different at pH 4.75 and 5.5. At pH 4.75, the precursor is derived from myoglobin and at pH 5.5 the precursor is derived heme biosynthetic pathway. Since large amount of myoglobin is assumed to exist in meat compared with the precursor(s) in heme biosynthesis, the mechanism at pH 4.75 is more possible than at 5.5. On the other hand, Parma ham is a long aged dry-cured ham. Many proteins are degraded during aging (Grossi et al, 2014) and

Table 5. Essential components for ZnPP and PPIX formation at pH 4.75 and 5.5 in pork

Components	ZnPP and PPIX formation	
	pH 4.75	pH 5.5
Insoluble fraction	O	O
Soluble fraction	O	O
<10 kDa fraction	O	O
Heat stable compounds	O	O
>10 kDa fraction	O	O
10-30 kDa fraction	O	O
Myoglobin	O	X
42 and/or 60 kDa proteins	X	O

myoglobin might also be degraded during prolonged manufacturing. If the degradation of myoglobin is the rate determining step at the pH 4.75 mechanism and many reactions of ZnPP formation are common at pH 4.75 and 5.5, large amount of ZnPP might be formed under pH condition of Parma ham. However, in the present study the reason could not be elucidated. If further contributors were identified, the mechanism of ZnPP formation will be elucidated.

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