Zn protoporphyrin IX is formed not from heme but from protoporphyrin IX.

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Abstract

We examined the effects of exogenous myoglobin, a bivalent chelator, and nitrite on Zn protoporphyrin IX (ZPP) formation by using model systems. ZPP was formed in a model solution without addition of exogenous myoglobin. After incubation, the amount of ZPP in a model solution was increased but that of heme was not decreased compared with the amounts before incubation. Protoporphyrin IX (PPIX) instead of ZPP also accumulated in a model solution with addition of EDTA, but the amount of heme was not reduced. These results suggested that ZPP was not formed by the Fe-Zn substitution in heme but was formed by the insertion of Zn into PPIX, which was formed independently. The fact that the effects of various factors in model systems with/without addition of a bivalent chelator were similar suggested that ZPP formation was strongly affected by PPIX formation. Inhibition of PPIX formation by nitrite might be the reason for the low levels of ZPP in cured meats.

Keywords: Zn protoporphyrin IX; protoporphyrin IX; color; dry-cured meat products; nitrite; nitric oxide

1. Introduction

Dry-cured meat products have been produced and consumed throughout history by a diversity of cultures in different parts of the world. Parma ham, one of the Italian dry-cured hams, is made from heavier pigs (> 150 kg in liveweight) and is processed for at least 12 months without addition of nitrite/nitrate (Toldrá, 2002). It has been reported that Parma ham has a red porphyrin derivative that is not nitrosylmyoglobin or oxymyoglobin and is extractable by water, acetone/water (75%/25%) and tetrahydrofuran (Morita, Niu, Sakata & Nagata, 1996; Møller, Adamsen & Skibsted, 2003; Parolari, Gabba & Saccani, 2003). The pigment was purified and identified to be Zn protoporphyrin IX (ZPP) by ESI-HR-MS analysis (Wakamatsu, Nishimura & Hattori, 2004). Moreover, the presence of zinc in the red pigment was directly demonstrated by scanning electron microscopy/energy dispersive X-ray microanalysis (SEM-EDX) (Wakamatsu, Ito, Nishimura & Hattori, 2007). Møller, Adamsen, Catharino, Skibsted, & Eberlin (2007) showed by using ESI-MS and TOF-MS analysis that zinc protoporphyrin IX is present not only in Parma ham but also in Iberian ham.

Effects of various factors on ZPP formation have been investigated using a model system, and it has been shown that oxygen inhibited ZPP formation and that ZPP was formed in the absence of microorganisms (Wakamatsu, Okui, Nishimura & Hattori, 2004). It has also been reported that the amount of ZPP and protoporphyrin IX (PPIX) were increased in pork and turkey during anaerobic conditions (Veberg et al., 2006). By use of autofluorescence of ZPP, it has been revealed that ZPP was distributed in not only lean meat but also fat tissue by using near-UV purple LED irradiation and an image analysis (Wakamatsu, Odagiri, Nishimura & Hattori, 2006).

On the other hand, exogenous myoglobin was added into a model solution that was established by Wakamatsu, Okui, Nishimura & Hattori (2004). The addition of exogenous myoglobin may affect ZPP formation. Moreover, the amount of ZPP formed

63 has only been measured by fluorescence intensity, and the amount of heme has not been
64 determined at all. In order to elucidate the mechanism by which ZPP is formed, these
65 porphyrins must be quantitatively determined. A recent study has shown that ZPP is
66 present not only in Parma ham but also in Iberian ham but that ZPP content in meat
67 products cured with nitrite is very low (Adamsen Møller, Laursen, Olsen & Skibsted,
68 2006). It is still not clear why ZPP content in cured meat products is much lower than
69 that in dry-cured ham without addition of nitrite.

70 In this study, to elucidate the mechanism by which ZPP is formed, we investigated
71 the effects of various factors on ZPP formation by using a simpler model system with no
72 addition of exogenous myoglobin and a new model system in which PPX was formed by
73 the addition of ethylenediaminetetraacetate (EDTA). Then we measured the contents
74 of heme, ZPP and PPIX before and after incubation in these model solutions and
75 investigated stoichiometrically the mechanism by which ZPP or PPIX was formed.
76 Additionally, we investigated the inhibitory effect on ZPP formation of nitrite, which is
77 generally used in cured meat products.

2. Materials and methods

2.1. Materials

Pork loin samples (n = 3) were purchased from various retail markets. Myoglobin (from horse skeletal muscle), penicillin G potassium, streptomycin sulfate and gentamicin sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetone, acetic acid, acetate ethyl, methanol and ammonium acetate were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Methanol was of HPLC grade and other chemicals were of analytical grade.

2.2. Model system

Model solutions were prepared as described by Wakamatsu, Okui, Nishimura & Hattori (2004). Experimental designs are shown in Table 1. Pork loin was homogenized with 2 volumes of distilled water using a homogenizer at 10,000 rpm for 1 min. Antibiotics were added to the model solutions to final concentrations of 100 units/ml for penicillin G potassium, 0.1 mg/ml for streptomycin sulfate and 0.05 mg/ml for gentamicin sulfate. The solutions were put into gas-impermeable bags and incubated at 25 ˚C for 5 days in darkness using an environmental chamber. An anaerobic condition was obtained by using a commercial kit (Oxygen Absorbing System; I.S.O. Inc., Yokohama, Japan) consisting of an oxygen absorber (A-500HS, I.S.O. Inc.) and an oxygen indicator tablet. The color of the oxygen indicator tablet changes from violet to pink when the oxygen concentration becomes less than 0.1%.

2.3. Fluorescent analysis

Extraction by acetone and fluorescent analysis were carried out as described by Wakamatsu, Okui, Nishimura & Hattori (2004) with minor modification. After extraction by acetone, the fluorescent spectra of the extracts were measured from 450 to 700 nm at 420/410 nm for excitation using a spectrofluorophotometer (RF-5300PC, Shimadzu Corp., Kyoto, Japan). Fluorescence intensity at 590 nm for excitation at 420 nm was regarded as the amount of ZPP 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 as much as possible.

2.4. Quantitative analysis of ZPP, PPIX and heme

The contents of ZPP, PPIX and heme were determined by HPLC as described by Guo, Lim & Peters (1991) with some modification. Porphyrins were extracted by acetic acid/acetate ethyl (1:4, v/v) as described by Smith, Doran, Mazur & Bush (1980) with some modification. One milliliter of model solution was mixed vigorously with 9 ml of acetic acid/acetate ethyl (1:4, v/v), and the mixture was held on ice for 30 min. After centrifugation (3,000 rpm, 15 min), the supernatant was collected. This extraction operation was carried out twice. The resulting solution was mixed with an equal volume of methanol/ammonium acetate (86:14, v/v, pH 5.16). The sample was filtered through a 0.45-μm filter (Minisart RC4, Sartorius AG, Goettingen, Germany). An STR ODS-II column (4.6 x 150 mm, Shinwa Chemical Industries Ltd., Kyoto, Japan) was used for separation of porphyrin, and the separation was carried out by isocratic elution using methanol/ammonium acetate (86:14, v/v, pH 5.16) at a flow rate of 0.6 ml/min at 35℃. Forty microliters of each sample was injected. The detection of ZPP and PPIX was carried out at excitation and emission wavelengths of 420/400 and 590/630 nm, respectively. Heme was monitored at 400 nm absorption. All operations of extraction

were carried out in darkness as much as possible.

2.5. Statistical analysis

Results are expressed as mean values of three independent trials in each individual. Data were analyzed using one-way analysis of variance (ANOVA) with Scheffé's test. Statistics were calculated using Microsoft Excel 2002 and a statistic add-in software (Excel Toukei 2002 for Windows, Social Survey Research Information Co., Ltd., Tokyo, Japan). A probability of $P<0.05$ was considered statistically significant.

3. Results and discussion

First, in order to investigate the effects of exogenous myoglobin in our established experimental model, the effect of the amount of added myoglobin on the amount of ZPP formed was examined (Fig. 1). When exogenous myoglobin was added at 0.1%, the amount of ZPP formed was maximal. With further addition of myoglobin, the amount of formed ZPP gradually decreased. The amount of ZPP formed was significantly increased with the addition of 0.1% exogenous myoglobin. The cause of the increase is unclear and it may be because myoglobin used in this study was metmyoglobin or included impurities. However, ZPP was formed despite no addition of myoglobin. Therefore, this result suggested that exogenous myoglobin is not always essential for the formation of zinc protoporphyrin IX and ZPP. It is also possible that ZPP is formed only from pork components. Since the addition of exogenous myoglobin in a model solution might make it difficult to elucidate the mechanism by which ZPP is formed, the mechanism should be investigated in a simpler model system with no addition of exogenous myoglobin. However, the cause of increase in ZPP formation by the addition of 0.1% exogenous myoglobin was not verified.

Next, we established a simpler model system composed of pork homogenate and antibiotics without addition of exogenous myoglobin and investigated the effects of various factors on ZPP formation (Fig. 2). Oxygen inhibited ZPP formation (Fig. 2A) and the amount of ZPP formed was increased with increase in the amount of pork added (Fig. 2B). The amount of ZPP formed rapidly increased during the first 3 days of incubation and increased slightly after the third day (Fig. 2C). The amount of ZPP formed was also increased with increase in incubation temperature within the range of temperatures examined (4, 15, 25 and 35˚C) (Fig. 2D). The amount of ZPP formed peaked at about pH 5.5 and decreased considerably at lower or higher pH (Fig. 2E).

166 The results showing that ZPP formation depended on many factors suggested the
167 involvement of an enzyme. The most notable dependence is the pH dependence. ZPP
168 formation peaked at 5.5, which is very close to the ultimate pH of meat. The optimal
169 pH range was narrower than those for general enzymes. Although the cause is not
170 clear, this pH range might be an important clue to elucidate the mechanism by which
171 ZPP is formed.

172 On the other hand, it has been reported that little ZPP was present in meat products
173 cured with nitrite and/or nitrate (Adamsen Møller, Laursen, Olsen & Skibsted, 2006).
174 Therefore, the influence of added nitrite was investigated by using the model system
175 (Fig. 2F). The formation of ZPP was inhibited significantly by addition of nitrite of
176 more than 10 μM (0.69 mg/L). Since the concentration at which ZPP formation was
177 inhibited was much lower than the amount of nitrite added to common meat products, it
178 was speculated that low ZPP content in meat products cured with nitrite and/or nitrate
179 is caused by the inhibition of ZPP formation by nitrite. However, the cause is still
180 unclear and future studies are needed.

181 In previous studies (Wakamatsu, Nishimura & Hattori, 2004; Wakamatsu, Okui,
182 Nishimura & Hattori, 2004), the amount of ZPP formed was determined by fluorescence
183 intensity and was not determined quantitatively. Heme content was also not
184 measured. Therefore, we determined the quantities of ZPP and heme in the model
185 solutions. As shown in Fig. 3, although the amount of ZPP had significantly increased
186 after 5-day incubation, a decrease in the amount of heme was not observed. PPIX was
187 not detected before and after incubation. Therefore, the total amount of ZPP and heme
188 was increased compared with the total amount before incubation. If ZPP was just
189 formed from heme with substitution of Zn for Fe, the sum total amount of heme and
190 ZPP would not be increased. This result therefore suggested that ZPP was not formed
191 by Fe-Zn substitution in heme but was independently formed in the model during
192 incubation. At the last step of heme biosynthesis, heme was formed by inserting Fe

193 into PPIX (Ferreira, 1999; Dailey, Dailey, Wu, Medlock, Rose & Wang, 2000; Dailey,
194 2002). It is therefore possible that ZPP is formed by insertion of Zn into PPIX
195 generated independently. Thus, the chelation of zinc by a chelator was predicted to
196 inhibit the formation of ZPP and to result in the accumulation of PPIX.
197 Next, we observed the fluorescence spectrum of acetone extract after incubation in
198 the model solution with addition of EDTA, a bivalent metal chelator (Fig. 4). A strong
199 peak at 630 nm was observed in the model solution with addition of EDTA. The
200 maximum excitation wavelength of the fluorescence peak was about 410 nm, shorter
201 than the excitation wavelength of ZPP (420 nm) in agreement with results of a previous
202 study (Shepherd & Dailey, 2005). This fluorescence peak coincided with that of PPIX
203 (Shepherd & Dailey, 2005; Veberg et al., 2006). Thus, since the chelation of zinc by
204 EDTA inhibited the formation of ZPP, ZPP seems to be formed by insertion of Zn into
205 PPIX.
206 Next, in order to determine whether PPIX was formed from the demetalation of
207 heme or independently, porphyrin contents in the model solutions with and without
208 addition of EDTA were determined after incubation (Fig. 5). Although the addition of
209 EDTA into a model solution significantly inhibited ZPP formation and the amount of
210 PPIX was increased significantly, there was no change in heme content ($P>0.05$). The
211 increase in PPIX content and the fact that the amount of heme did not change strongly
212 suggested that PPIX was formed not from heme but from another substrate. EDTA
213 was added into a model solution, but only a small amount of ZPP was present in the
214 solution. This is due to the presence of ZPP originally contained in pork (see Fig. 3).
215 We also investigated the effects of various factors on the formation of PPIX in simple
216 model systems with addition of EDTA. Oxygen tended to inhibit the formation of PPIX
217 (Fig. 6A). PPIX was increased with increase in pork content in model solutions (Fig.
218 6B). The amount of PPIX formed increased rapidly during the first 3 days of
219 incubation and increased slightly after the third day (Fig. 6C). The amount of PPIX

220 increased with increase in incubation temperature (Fig. 6D) and was maximal at about
221 pH 5.5 (Fig. 6E). The addition of more than 10 μM of nitrite inhibited ZPP formation
222 significantly (Fig. 6F). All of these patterns of PPIX formation are very similar to
223 those of ZPP formation (Fig. 2). Thus, these results indicated that ZPP in a model
224 solution was formed by the insertion of Zn into PPIX, which was formed not from heme
225 but independently. It is therefore possible that ZPP is also formed by the same
226 pathway in Parma ham. PPIX is formed from protoporphyrinogen IX (proto'gen) by
227 protoporphyrinogen oxidase (PPO; EC 1.3.3.4) in the heme biosynthesis system (Dailey,
228 2002). The enzyme activity of mammalian PPO has been shown to be maximal at pH
229 8.6 to 8.7 (Poulson, 1976), much higher than results obtained in the present study. On
230 the other hand, the inhibition of PPIX formation by oxygen was reduced in model
231 systems with addition of EDTA, but there was no significant difference between them.
232 There are two forms of PPO, an oxygen-dependent form and an oxygen-independent
233 form (Dailey, 2002), and they may contribute to PPIX formation. Our results revealed
234 that nitrite inhibited the formation of not only ZPP but also PPIX. Anaerobic oxidation
235 of proto'gen to PPIX has been demonstrated in extracts of several microorganisms by
236 nitrite or nitrate as an electron acceptor (Jacobs & Jacobs, 1976; Klemm & Barton,
237 1985). Since the inhibition of PPIX formation by nitrite is not still clear, further
238 studies are needed to elucidate the contribution of proto'gen and PPO to ZPP/PPIX
239 formation. The patterns of ZPP and PPIX formation were similar in model systems.
240 The pH curves with maximum peaks at about 5.5 are noteworthy, and they might serve
241 as a clue to elucidating the mechanism by which ZPP/PPIX is formed.

242 Furthermore, our results revealed that nitrite inhibited the formation of not only
243 ZPP but also PPIX. Color formation in cured meat products is caused mainly by the
244 reaction of endogenous myoglobin in meat with nitric oxide (NO) (Sakata, 2000; Fox,
245 1966). NO is produced from added nitrite (or nitrate) as the curing agent. NO has a
246 high affinity for non-heme iron proteins (Fujii & Yoshimura, 1996) and copper proteins

247 (Suzuki, 1996). NO is a paramagnetic molecule and has a high affinity for metal ions
248 other than heme (Fujii and Yoshimura, 1996). Consequently, many metalloenzymes
249 are inactivated by NO. Iron-sulfur (Fe-S) proteins have a very high affinity for NO.
250 Mammalian ferrochelatase contains a [2Fe-2S] cluster (Ferreira, Franco, Lloyd, Pereira,
251 Moura, Moura, & Huynh, 1994; Dailey, Dailey, Wu, Medlock, Rose & Wang, 2000), and
252 the cluster is labile and sensitive to destruction by NO (Sellers, Johnson, & Dailey,
253 1996). It has been reported that ferrochelatase as a zinc chelatase participate in ZPP
254 formation from myoglobin in pork loin extract and porcine heart extract (Ishikawa,
255 Yoshihara, Baba, Kawabuchi, Sato, Numata & Matsumoto, 2006a; Ishikawa, Yoshihara,
256 Baba, Kawabuchi, Sato, Numata & Matsumoto, 2006b). Although the participation of
257 ferrochelatase is still not clear, the inactivation of metalloenzyme by NO may be
258 involved in the inhibition of PPIX formation.
259 The results of this study might serve as a clue to elucidating the mechanism by which
260 ZPP/PPIX is formed. Since the formation of PPIX had a strong influence on that of ZPP,
261 it is necessary to investigate the steps prior to PPIX formation in more detail.
262

4. Conclusion

This study demonstrated that ZPP in a model solution was not formed by Fe-Zn substitution in heme but was formed by the insertion of Zn into PPIX, which was formed independently. It was shown that ZPP formation was strongly influenced by PPIX formation. The fact that there is little ZPP in cured meat products is because ZPP cannot be formed as a result of inhibition of PPIX formation by nitrite. A full understanding of ZPP formation in Parma ham awaits elucidation of the formation of PPIX or its precursor.

Acknowledgments

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279 References

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364 Figure legends

365

366

367 Fig. 1. Effect of exogenous myoglobin addition on the formation of ZPP. Model
368 solutions with addition of various amounts of exogenous myoglobin were incubated
369 for 5 days at 25℃ anaerobically. The fluorescence intensity of acetone extracts was
370 measured. Bars represent the standard deviation of the means (n=3). [ab]: Values
371 bearing different letters are significantly different ($P < 0.05$).

372

373 Fig. 2. Effects of oxygen (A), pork content (B), incubation day (C), temperature (D), pH
374 (E) and nitrite (F) on the formation of ZPP in model solutions with no addition of
375 exogenous myoglobin. The fluorescence intensity of acetone extracts was measured
376 after incubation. Bars represent the standard deviation of the means (n=3). [abcde]:
377 Values in the same examination bearing different letters are significantly different
378 ($P < 0.05$).

379

380 Fig. 3. Heme (■) and ZPP(■) concentrations in model solutions without addition of
381 exogenous myoglobin before and after incubation. Porphyrins extracted by acetic
382 acid/acetic ethyl were measured by HPLC. Bars represent the standard deviation
383 of the means (n=3). n.s.: not significant (vs before incubation).

384

385 Fig. 4. Fluorescence pattern of acetone extract of model solutions with (broken line, Ex.
386 410 nm) and without (solid line, Ex. 420 nm) addition of EDTA after 5 days at 25℃.

387

388 Fig. 5. Heme (■), ZPP (■) and PPIX (□) concentrations in model solutions with and

without addition of EDTA after 5 days at 25℃. Porphyrins extracted by acetic acid/acetic ethyl were measured by HPLC. Bars represent the standard deviation of the means (n=3). n.s.: not significant (vs with addition of EDTA).

Fig. 6. Effects of oxygen (A), pork content (B), incubation day (C), temperature (D), pH (E) and nitrite (F) on the formation of ZPP in the model solution with addition of EDTA. The fluorescence intensity of acetone extracts was measured after incubation. Bars represent the standard deviation of the means (n=3). ^{abcde}: Values in the same examination bearing different letters are significantly different ($P < 0.05$). n.s.: not significant.

Table 1. Experimental designs by using a model system

Figure1

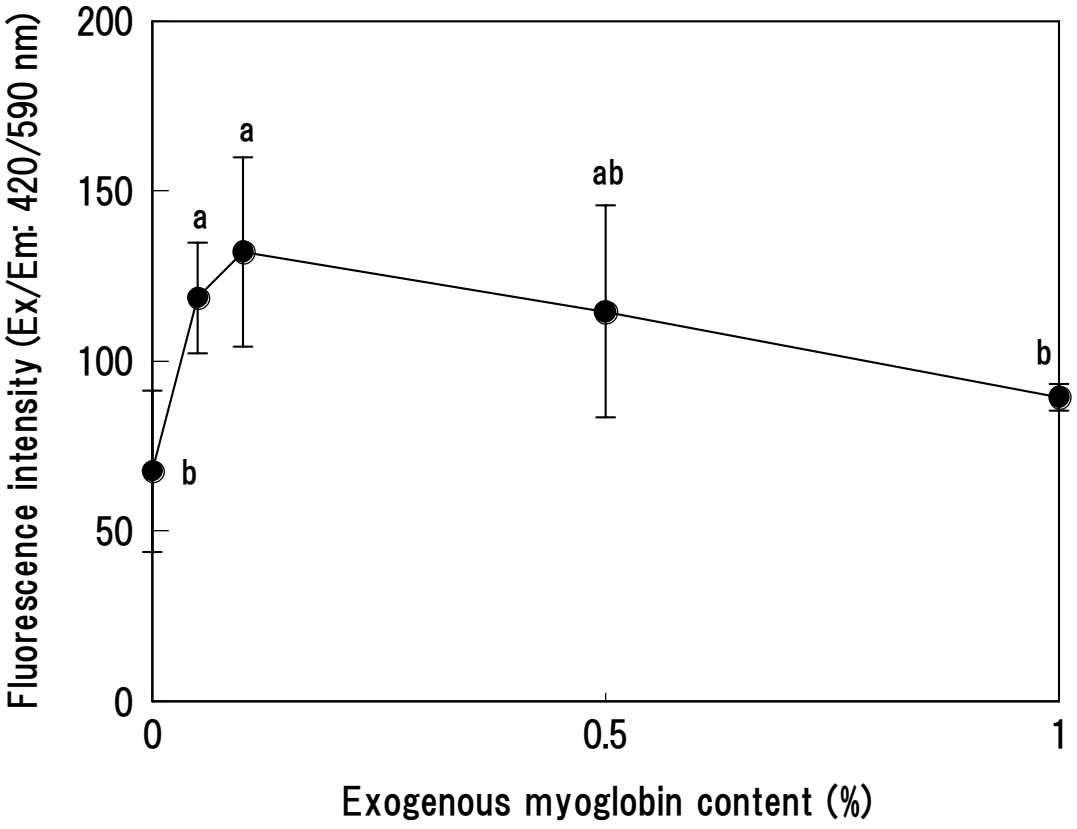

Fig.1

Figure2

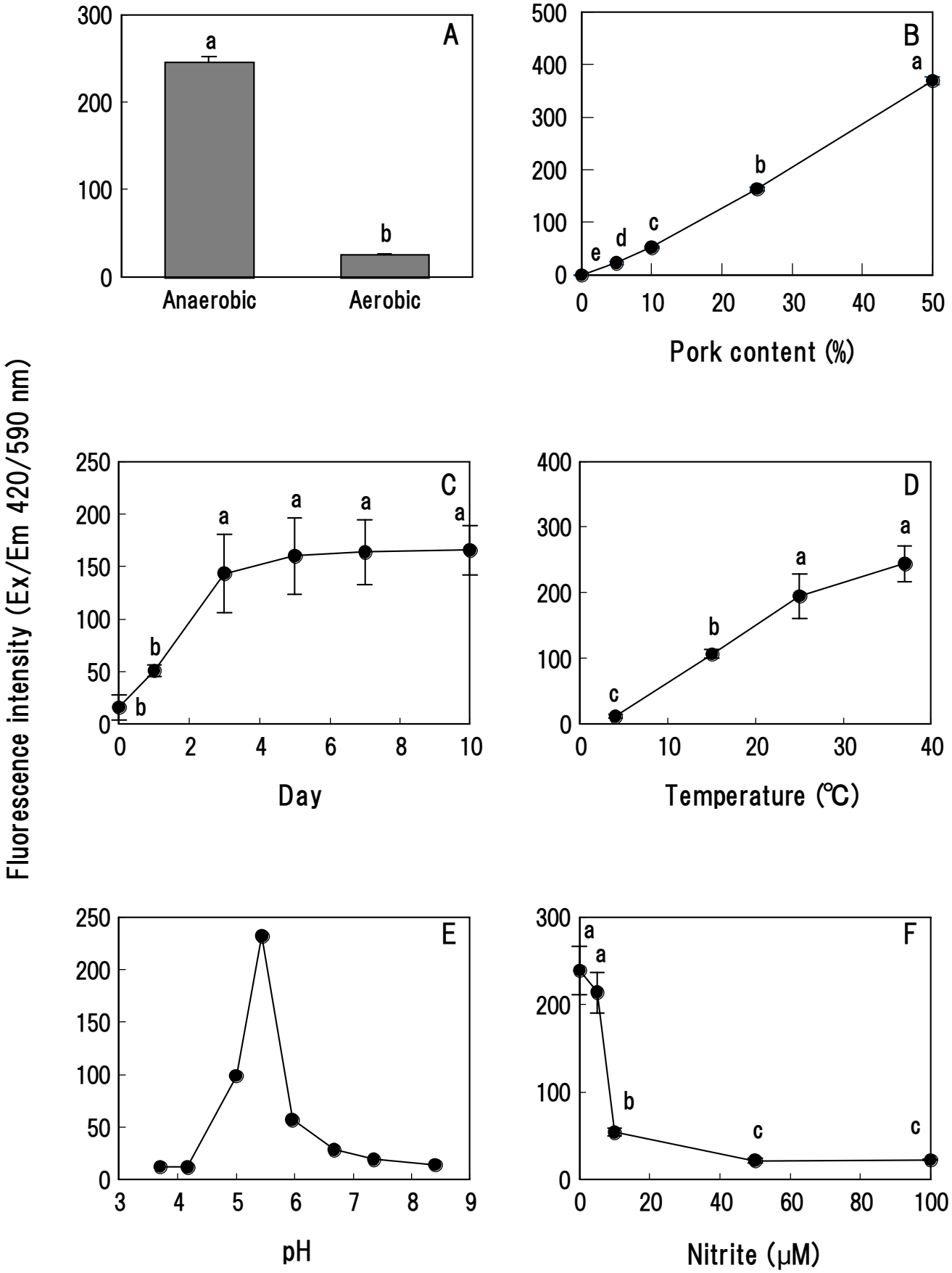

Fig.2

Figure3

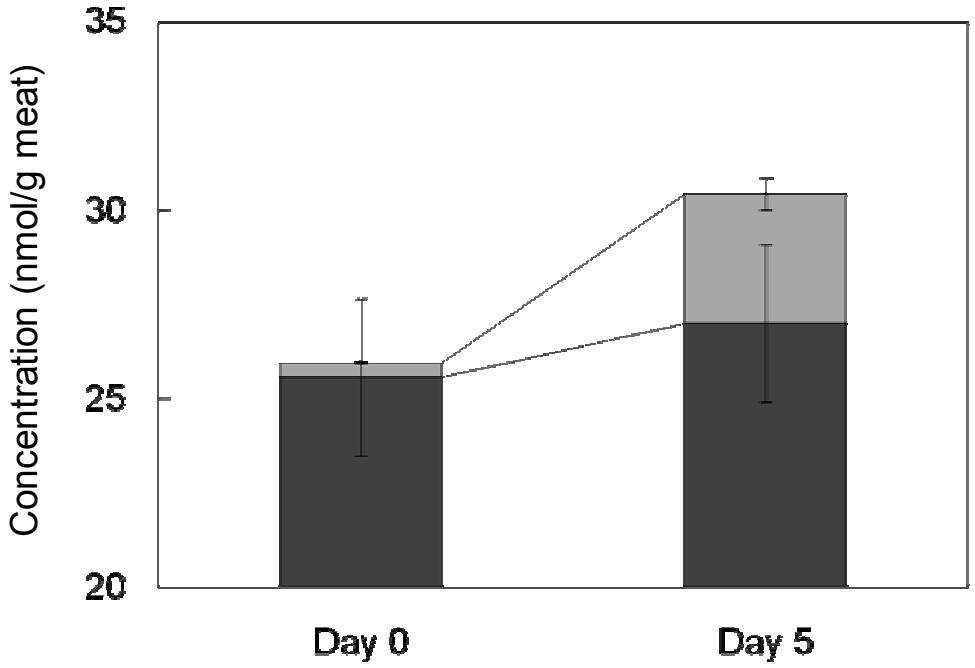

Fig. 3

Figure4

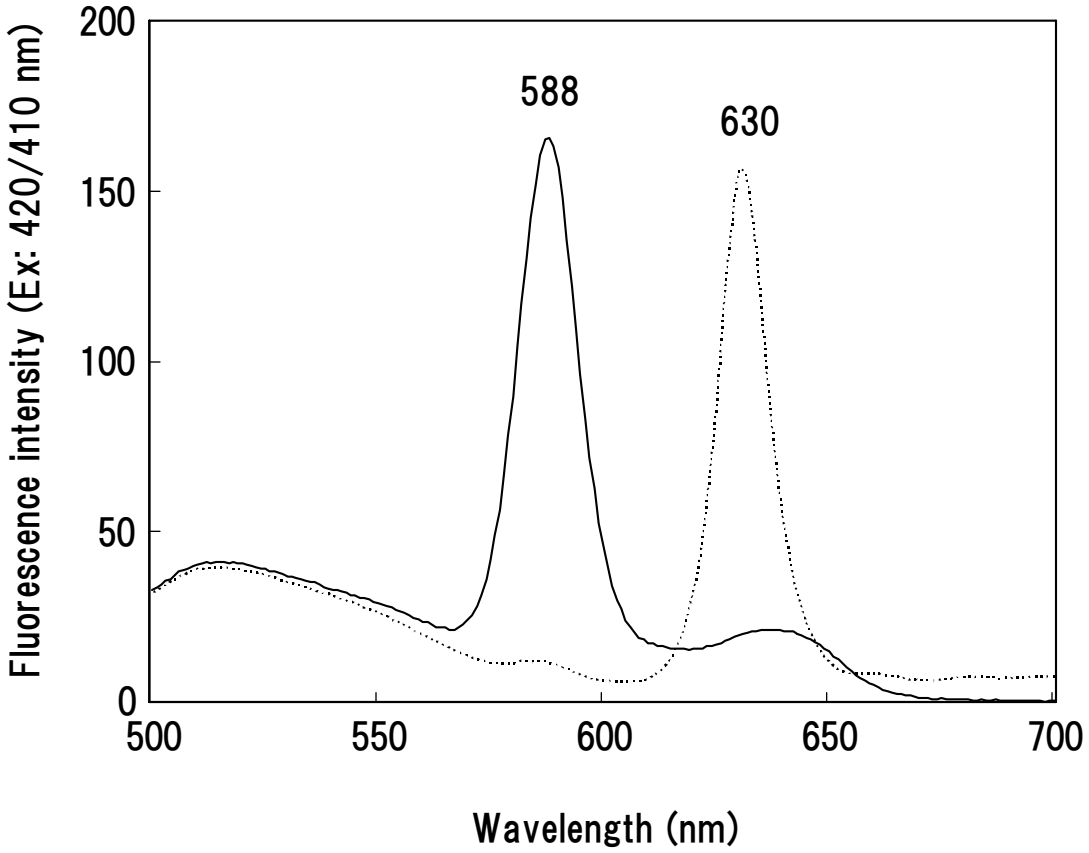

Fig.4

Figure5

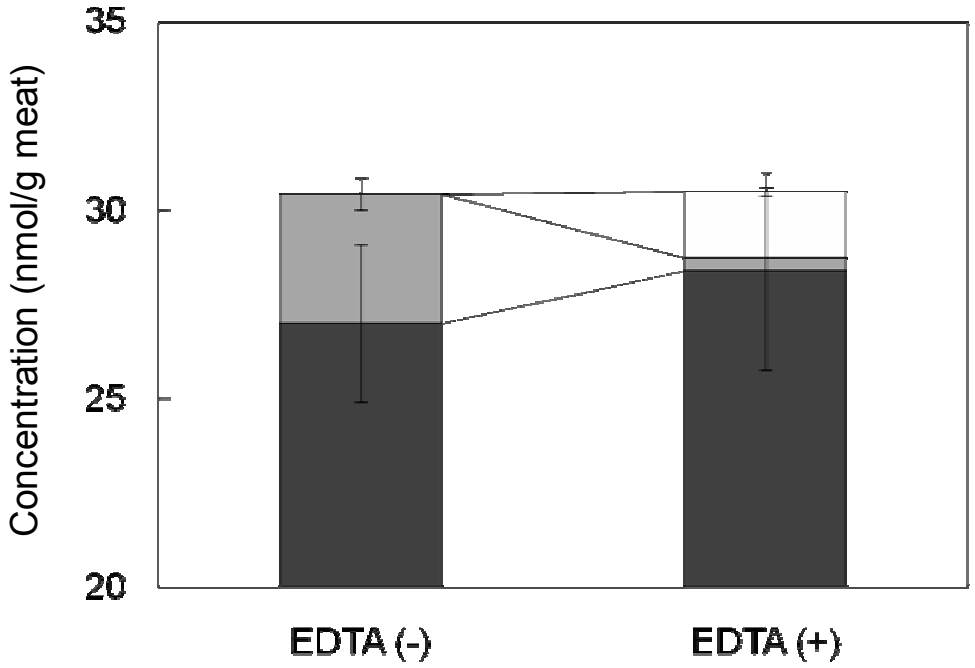

Fig. 5

Figure6

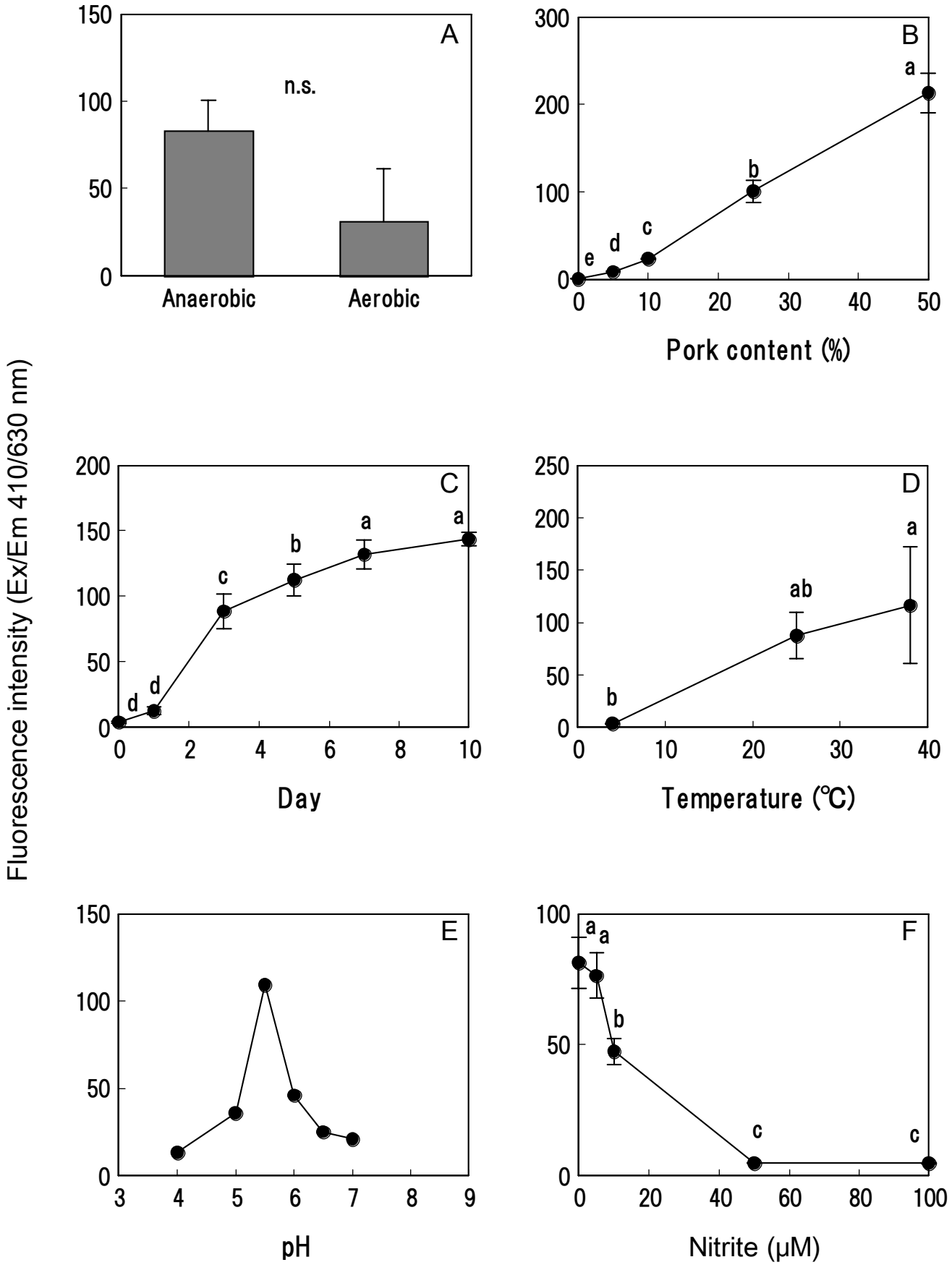

Fig.6

Table1

Table 1. Experimental designs by using a model system

Pork	Myoglobin	EDTA	Oxygen	Temperature	Day	pH	Nitrite
Experimental model designs with addition of exogenous myoglobin							
20%	0-1%[a]	-	< 0.1%	25ºC	5	-	-
Experimental model designs without addition of exogenous myoglobin							
20%	-	-	< 0.1%	25ºC	5	-	-
20%	-	-	NA[b]	25ºC	5	-	-
0-50%[c]	-	-	< 0.1%	25ºC	5	-	-
20%	-	-	< 0.1%	25ºC	0-10[d]	-	-
20%	-	-	< 0.1%	4-37ºC[e]	5	-	-
20%	-	-	< 0.1%	25ºC	5	3.5-8.5	-
20%	-	-	< 0.1%	25ºC	5	-	0-100 μM[f]
Experimental model designs in the chelation on bivalent metals by EDTA							
20%	-	0.5 mM	< 0.1%	25ºC	5	-	-
20%	-	0.5 mM	NA[b]	25ºC	5	-	-
0-50%[c]	-	0.5 mM	< 0.1%	25ºC	5	-	-
20%	-	0.5 mM	< 0.1%	25ºC	0-10[d]	-	-
20%	-	0.5 mM	< 0.1%	4, 25, 37ºC	5	-	-
20%	-	0.5 mM	< 0.1%	25ºC	5	3.5-8.5	-
20%	-	0.5 mM	< 0.1%	25ºC	5	-	0-100 μM[f]

Antibiotics were added to all model solutions to final concentrations of 100 units/ml for penicillin G potassium, 0.1 mg/ml for streptomycin sulfate and 0.05 mg/ml for gentamicin sulfate.
[a]; 0, 0.05, 0.1, 0.5, 1%: [b]; no adjusted: [c]; 0, 5, 10, 25, 50%: [d]; 0, 1, 3, 5, 10 day: [e]; 4, 15, 25, 37ºC: [f]; 0, 5, 10, 50, 100 μM