Establishment of a model experiment system to elucidate the mechanism by which Zn-protoporphyrin is formed in nitrite-free dry-cured ham

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Abstract

The aim of this study was to establish a model experiment system to elucidate the mechanism by which Zn-protoporphyrin (ZPP) is formed in Parma ham. The established model consisted of myoglobin, meat and antibiotics, and incubation under anaerobic conditions resulted in a greater yield of ZPP. Formation of ZPP was observed even in the presence of various antiseptics. The amount of ZPP formed increased as the period of incubation increased. ZPP formation was inhibited by heating meat homogenate depending on the heating temperature. Our results show that anaerobic conditions are suitable for the formation of ZPP in meat products without nitrate or nitrite and that endogenous enzymes as well as microorganisms may be involved in ZPP formation.

Keywords: Parma ham, Zn-protoporphyrin IX, heme, microorganism

1. Introduction

Salting have been used as a means of the meat preservation from ancient times and various dry-cured meat products adapted to various climates have been uniquely developed in various places over many years (Toldrá, 2002a; Flores, 1997). Nitrate and/or nitrite are now used in many meat products to improve color. In Italian Parma ham, however, the use of nitrate or nitrite is banned. Parma hams are manufactured by using only pork and salt (Toldrá, 2002b). The inside color of the ham is bright red. The red pigment has not been identified and, unlike known myoglobin derivatives, is stable to light (Morita, Niu, Sakata, & Nagata, 1996). Parolari, Gabba and Saccani (2003) reported that dry curing of meat without nitrate yields a lipophylic stable red pigment whose hydrophobicity increases with ageing. We have recently identified the pigment extracted from Parma ham as being Zn-protoporphyrin IX (ZPP), in which the iron in heme is substituted by zinc (Wakamatsu, Nishimura, & Hattori, 2004).

Construction of a model experiment system would be useful for elucidation of the mechanism by which ZPP is formed in Parma ham. Simple composition and long incubation period would be important to model the manufacturing of Parma ham. Composition of only meat and myoglobin is the simplest, but the effects of microorganisms during a long incubation period can not be ignored. Morita, Niu, Sakata and Nagata (1996) reported that the Parma ham pigment is formed by staphylococci. Many studies have shown that microorganisms change the color of meat and meat products (Kalchayanand, Ray, Field & Johnson, 1989; Faustman Johnson, Cassens & Doyle, 1990; Arihara, Kushida, Kondo, Itoh,

Luchansky & Cassens, 1993). However, considering the low microbial counts and the difficult conditions for microbial growth in the interior of dry-cured hams (Toldrá & Etherington, 1988; Toldrá, 1998), it is unlikely that only microorganisms contribute to the formation of ZPP in Parma ham. Additionally, the oxygen pressure in the interior of meat products is the low oxygen pressure. Effects of oxygen can not be ignored considering the high reactivity between heme and oxygen. In this study, we developed a model experiment system to elucidate the mechanism by which ZPP is formed during the process of traditional dry-cured ham manufacturing, and we investigated the effect of oxygen on and the involvement of microorganisms and endogenous enzymes in ZPP formation.

2. Materials and Methods

2.1. Materials

Pork loin samples were purchased from various retail markets. Myoglobin (from horse skeletal muscle), penicillin G potassium, streptomycin sulfate, gentamicin sulfate and sodium azide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Thimerosal was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used in this study were of analytical grade.

Penicillin and streptomycin were dissolved in sterilized distilled water at 10,000 units/ml and 10 mg/ml, respectively, and were kept frozen at -20 ˚C after

filtration sterilization until use. Gentamicin was dissolved in sterilized distilled water at 10 mg/ml and was kept frozen at -20 ˚C after filtration sterilization until use.

2.2. Model experiment

Model solutions consisted of myoglobin (final concentration of 0.1%) and pork homogenates (final meat concentration of 20%) with or without preservatives. About 20 grams of pork loin was homogenized with 2 volumes of distilled water using a homogenizer (Cell Master CM-100, Az One Co., Tokyo, Japan) 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. Sodium azide and thimerosal were added to the model solutions to final concentrations of 0.01%. The solutions were put into gas-impermeable bags and incubated at 25 ˚C for 5 days. 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%.

For heat tests, pork homogenates and distilled water mixtures were heated in a hot bath for 30 minutes after each setting temperature (40-80 ˚C) had been reached. After cooling in water, myoglobin (final concentration of 0.1%) and antibiotics (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) were added to the samples, and the samples were anaerobically incubated at 25 ˚C for 5 days.

2.3. Fluorescent analysis

One milliliter of incubated solution was mixed well with 3 ml of ice-cooled acetone, and the mixture was held on ice for 30 minutes. After filtration through filter paper (No.5C, Toyo Roshi Co., Ltd., Tokyo, Japan), the fluorescent spectra of the extracts were measured from 500 to 700 nm at 420 nm for excitation using a Model 650-60 fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) (Wakamatsu, Nishimura, & Hattori, 2004). Fluorescence intensity at 590 nm for excitation at 420 nm was regarded as the amount of ZPP formed. All operations were carried out under shading as much as possible.

2.4. Microbiological analysis

One milliliter of model solution was mixed with 9 ml sterile phosphate-buffered saline (PBS). Serial 10-fold dilutions were prepared by diluting 1 ml in 9 ml sterile PBS. Two duplicate sheets were prepared from each dilution by pouring 1 ml onto Sanita-kun Aerobic Count sheets (Chisso Corp., Tokyo, Japan) (Morita, Ushiyama, Aoyama, & Iwasaki, 2003). The sheets were incubated at 37 ˚C for 48 h. Counts were determined from sheets bearing 30-300 colonies. Microbial counts are expressed in colony-forming units per milliliter (CFU/ml).

3. Results and discussion

Simple composition is desirable for the establishment of a model experiment system to elucidate the mechanism of ZPP formation in Parma ham. The system used in this study was composed of only 0.1% myoglobin and 20% pork. The amount of added pork was determined from the ease of handling in preliminary experiments. Since the oxygen partial pressure inside large-sized meat products is remarkably low, we first investigated the influence of oxygen in our model experiment system. Fluorescent spectra of 75% acetone extracts of model solutions, consisting of only myoglobin and pork, after aerobic or anaerobic incubation for 5 days at 25˚C are shown in Fig. 1. When the model solution was anaerobically incubated, strong and weak fluorescent peaks at about 590 and 640 nm were observed. This fluorescent spectrum is consistent with the ZPP spectrum obtained in our previous study (Wakamatsu, Nishimura, & Hattori, 2004). On the other hand, strong fluorescence with a maximum peak at <500 nm was emitted when the solution was aerobically incubated. A fluorescent peak was observed at about 590 nm, but they were overlapped. Although the strong fluorescence with a maximum peak at <500 nm is unidentifiable, since it was not observed in Parma ham (Wakamatsu, Nishimura, & Hattori, 2004), it is thought that an anaerobic condition is suitable for ZPP formation.

Next, we examined the effect of incubation period. Model solutions that each consisted of 0.1% myoglobin and 20% pork were anaerobically incubated at 25 ˚C

for various periods, and then fluorescent intensities (Ex/Em 420/590 nm) of 75% acetone extracts of model solutions were measured (Fig. 2). ZPP was not formed after 1-day incubation, but the amount of ZPP dramatically increased thereafter. The amount of ZPP increased only gradually after the seventh day of incubation. We therefore decided that 5-7 days was the most suitable period for incubation in our model experiment system.

Since there is a possibility of microbiological effects in our experiment system, we investigated the effects of antiseptics. Model solutions consisting of 0.1% myoglobin, 20% pork and various antiseptics were anaerobically incubated for 5 days at 25 ˚C. As shown in Fig. 3, the amount of ZPP formed in the presence of thimerosal was the greatest and that in the presence of sodium azide was the smallest. Formation of ZPP was observed in the presence of each of the antiseptics used in this study. Many microorganisms survived after incubation for five days without antiseptics ($>10^8$). However, in the presence of sodium azide or antibiotics, the microorganisms did not survive after incubation. On the other hand, the addition of thimerosal to the model solution did not result in death of microorganisms after incubation (4.7×10^4). Sodium azide, which is one of the respiratory and electron transport inhibitors, has been widely used as an antiseptic in many biochemical experiments. However, since it bonds with heme iron of many heme proteins, it may have some of influence on ZPP formation, i.e. Zn-Fe substitution in heme. Thimerosal has also been widely used as an antiseptic in many biochemical experiments, and it inactivates many enzymes by bonding with thiol-groups. Although both are effective as antiseptics, their antibacterial mechanism may have an effect on Zn-Fe substitution. Penicillin

blocks the formation of bacterial cell walls and has an antibacterial effect on Gram-positive bacteria. Streptomycin and gentamycin inhibit initiation, elongation and termination of protein synthesis in prokaryotes and induce misreading. They have antibacterial effect on both Gram-negative and Gram-positive bacteria. There is no cell wall in animal cells, and proteins are not synthesized because meat is skeletal muscle after death. It is therefore probable that these antiseptics have no effect on Zn-Fe substitution in our model. Since these antibiotics act not bacteriostatically but bactericidally and since microorganisms were not detected in model solutions after incubation for 5 days, it appears that surviving microorganisms have little effect on Fe-Zn substitution. We established a model experiment system that consists of meat, Mb and antibiotics were anaerobically incubated.

Next, we examined the effects of oxygen on the formation of ZPP in the presence of antibiotics. Fig. 4 shows fluorescent spectra of 75% acetone extracts of model solution after anaerobic or aerobic incubation for 5 days at 25 ˚C. The characteristic fluorescence spectrum pattern of ZPP was observed after anaerobic incubation in the presence of antibiotics. On the other hand, little ZPP was formed after aerobic incubation. The fluorescent intensities were remarkably increased by removal of oxygen. These results suggest that oxygen inhibits the formation of ZPP. Therefore, something other than microorganisms that induces the formation of ZPP might exist in meat.

Since ZPP was formed in the presence of any of the antiseptics used in this study, it is necessary to consider the effects of factors other than the action of microorganisms. Therefore, we investigated the effects of pre-heating of meat on

ZPP formation. The amounts of ZPP formed at different heating temperatures are shown in Fig. 5. In the control experiment, a considerable amount of ZPP was formed after incubation for five days. On the other hand, the amount of ZPP was reduced by heating at 40 ˚C for 30 minutes, and the amount of ZPP decreased with rise in heating temperature. Heating at 60 ˚C or higher resulted in equivalent degrees of fluorescence intensity before and after incubation and almost no formation of ZPP. It is not clear whether fluorescent intensity is reduced by heating at 40 ˚C, a temperature equivalent to body temperature. Since formation of ZPP in Parma ham differs from the reaction in a living body, it may be caused by unknown mechanism.

Zn is nonenzymatically inserted into porphyrins by reaction of a salt in an acidic or basic medium (Sanders et al., 2000). However, the reduction in the amount of ZPP formed by heating the meat homogenate is thought to be due to a substitution reaction by protein, not metal ions. Microbial counts in the interior of dry-cured hams are low, and conditions are not favorable for microbial growth (Toldrá, 1998; Toldrá & Etherington, 1988). It is conceivable that the ZPP formation from heme is caused by the action of meat endogenous enzymes. Actually, it has been reported that enzymes catalyzing the formation of ZPP exist in most organisms (Labbe, Vreman & Stevenson, 1999; Dailey & Dailey, 2003). However, it is not clear whether the activities of those enzymes are maintained during the long manufacturing process of Parma ham.

We established a model experiment system in which ZPP was formed by incubating myoglobin, meat and antibiotics in the absence of oxygen. Since, however, the antibiotics used have no effect on fungi and yeast, it is unlikely that

all microorganisms were controlled in this study. Further studies are needed to elucidate the mechanism of Fe-Zn substitution during the manufacturing process of Parma ham.

4. Conclusions

ZPP, a nitrate and/or nitrite-free dry-cured ham pigment, could be formed in a simple model. Oxygen inhibited the formation of ZPP. The finding that ZPP was formed even in the presence of antiseptics, the fact that microbial counts inside hams are low and that the conditions are not favorable for microbial growth, and the finding of reduction in the amount of ZPP formed by heating meat indicate that not only microorganisms contribute to the formation of ZPP in nitrate and/or nitrite-free dry-cured meat products.

Reference

Arihara, K., Kushida, H., Kondo, Y., Itoh, M., Luchansky, J. B., & Cassens, R. G. (1993). Conversion of metmyoglobin to bright red myoglobin derivatives by *Chromobacterium violaceum, Kurthia* sp., and *Lactobacillus fermentum* JCM1173. *Journal of Food Science, 58*, 38-42.

Dailey, H. A., & Dailey, T. A. (2003). Ferrochelatase In K. M. Kadish, K. M. Smith, & R. Guilard, *The Porphyrin Handbook* (Vol. 12, pp. 93-121). San Diego: Academic Press.

Faustman, C., Johnson, J. L., Cassens, R. G., & Doyle, M. P. (1990). Color reversion in beef. Influence of psychrotrophic bacteria. *Fleischwirtschaft*, *70*, 676-679.

Flores, J. (1997). Mediterranean vs northern European meat products. Processing technologies and main differences, *Food Chemistry, 59*, 505-510.

Kalchayanand, M., Ray, B., Field, R. A., & Johnson, M. C. (1989). Spoilage of vacuum-packaged refrigerated beef by *Clostridium*. *Journal of Food Protection, 52*, 424-426.

Labbe, R. F., Vreman, H. J., & Stevenson, D. K. (1999). Zinc protoporphyrin: A metabolite with a mission, *Clinical Chemistry, 45*, 2060-2072.

Morita, H., Niu, J., Sakata, R., & Nagata, Y. (1996). Red pigment of Parma ham and bacterial influence on its formation. *Journal of Food Science*, *61*, 1021-1023.

Morita H., Ushiyama M., Aoyama S. & Iwasaki M. (2003). Sensitivity and specificity of the Sanita-kun Aerobic Count: Internal validation and independent laboratory study, *Journal of AOAC International, 86*, 355-366.

Parolari, G., Gabba L., & Saccani G. (2003). Extraction properties and absorption spectra of dry cured hams made with and without nitrate, *Meat Science*, *64*, 483-490.

Sanders, J. K. M., Bampos, N., Clyde-Watson, Z., Darling, S. L., Hawley, J. C., Kim, H.- J., Mak, C. C., & Webb, S. J. (2000). Axial Coordination Chemistry of Metalloporphyrins. In K. M. Kadish, K. M. Smith, & R. Guilard, *The Porphyrin Handbook* (Vol. 3, pp. 1-48). San Diego: Academic Press.

Toldrá, F. (2002a). A historical perspective. *Dry-cured meat products* (pp. 1-5).

Connecticut: Food & Nutrition Press.

Toldrá, F. (2002b). Manufacturing of dry-cured ham. *Dry-cured meat products* (pp. 27-62). Connecticut: Food & Nutrition Press.

Toldrá, F. (1998). Proteolysis and lipolysis in flavour development of dry-cured meat products, *Meat Science, 49*, S101-S110.

Toldrá, F. & Etherington, D. J. (1988). Examination of cathepsins B, D, H and L activities in dry-cured hams, *Meat Science, 23*, 1-7.

Wakamatsu, J., Nishimura, T., & Hattori, A. (2004). A Zn-porphyrin complex contributes to bright red color in Parma ham. *Meat Science, 67*, 95-100.

Figure & Table Legends

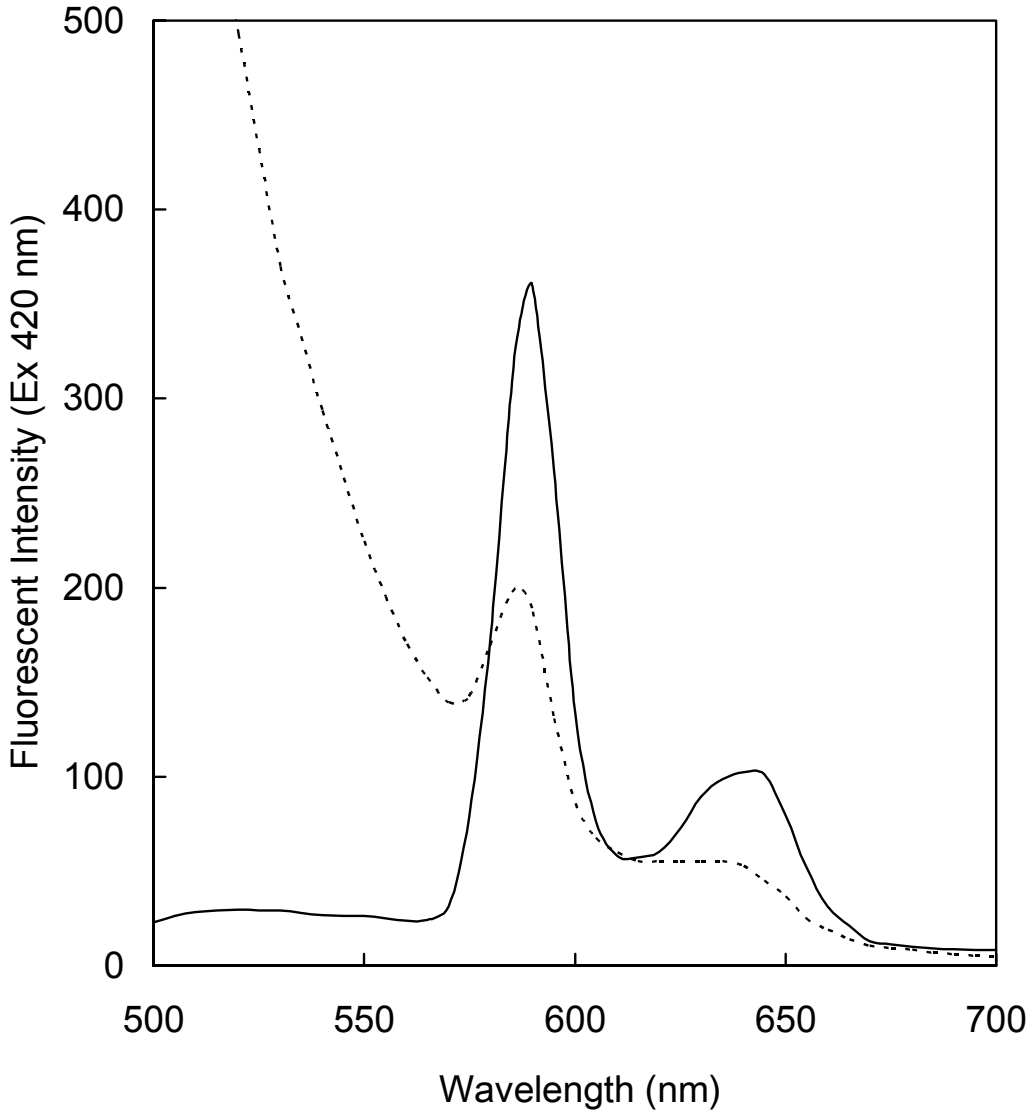
Fig. 1. Fluorescence pattern of acetone extract of model solution after anaerobic (——) and aerobic (- - - -) incubation for 5 days at 25 ˚C.

Fig. 2. Effects of anaerobic incubation time on the formation of ZPP.

Fig. 3. Effects of various antiseptics (sodium azide, 0.01%; antibiotics [100 units/ml penicillin G potassium, 0.1 mg/ml streptomycin sulfate and 0.05 mg/ml gentamicin sulfate]; thimerosal, 0.01%) on the formation of ZPP after anaerobic incubation for 5 days at 25 ˚C.

Fig. 4. Fluorescence pattern of acetone extract of model solution after anaerobic (——) and aerobic (- - - -) incubation for 5 days at 25 ˚C with antibiotics.

Fig. 5. Effects of pre-heating of the meat homogenate on the formation of ZPP before (0 days) and after anaerobic incubation (5 days) at 25 ˚C.



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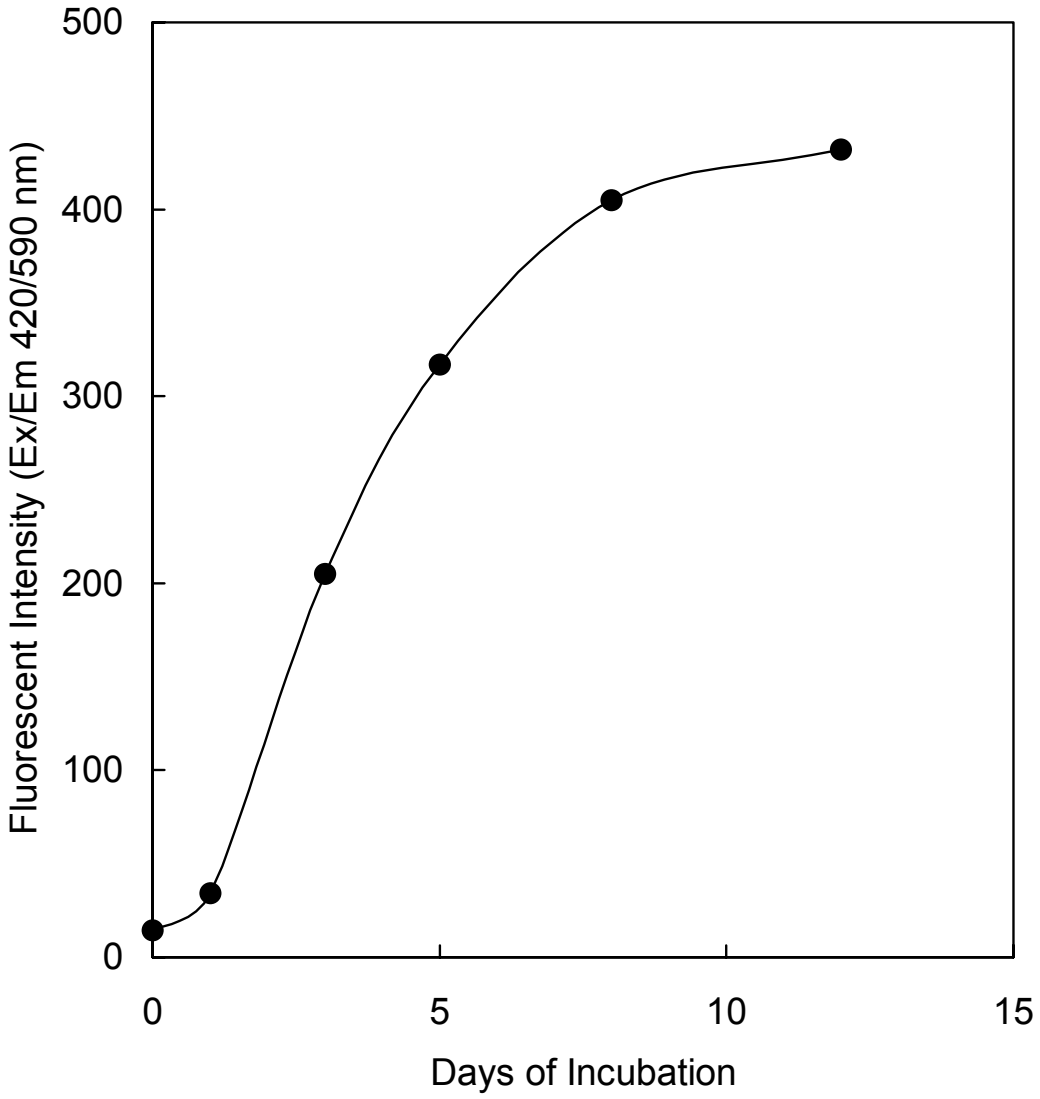
Fluorescence pattern of acetone extract of model solution after anaerobic (——) and aerobic (----) incubation for 5 days at 25 ˚C.



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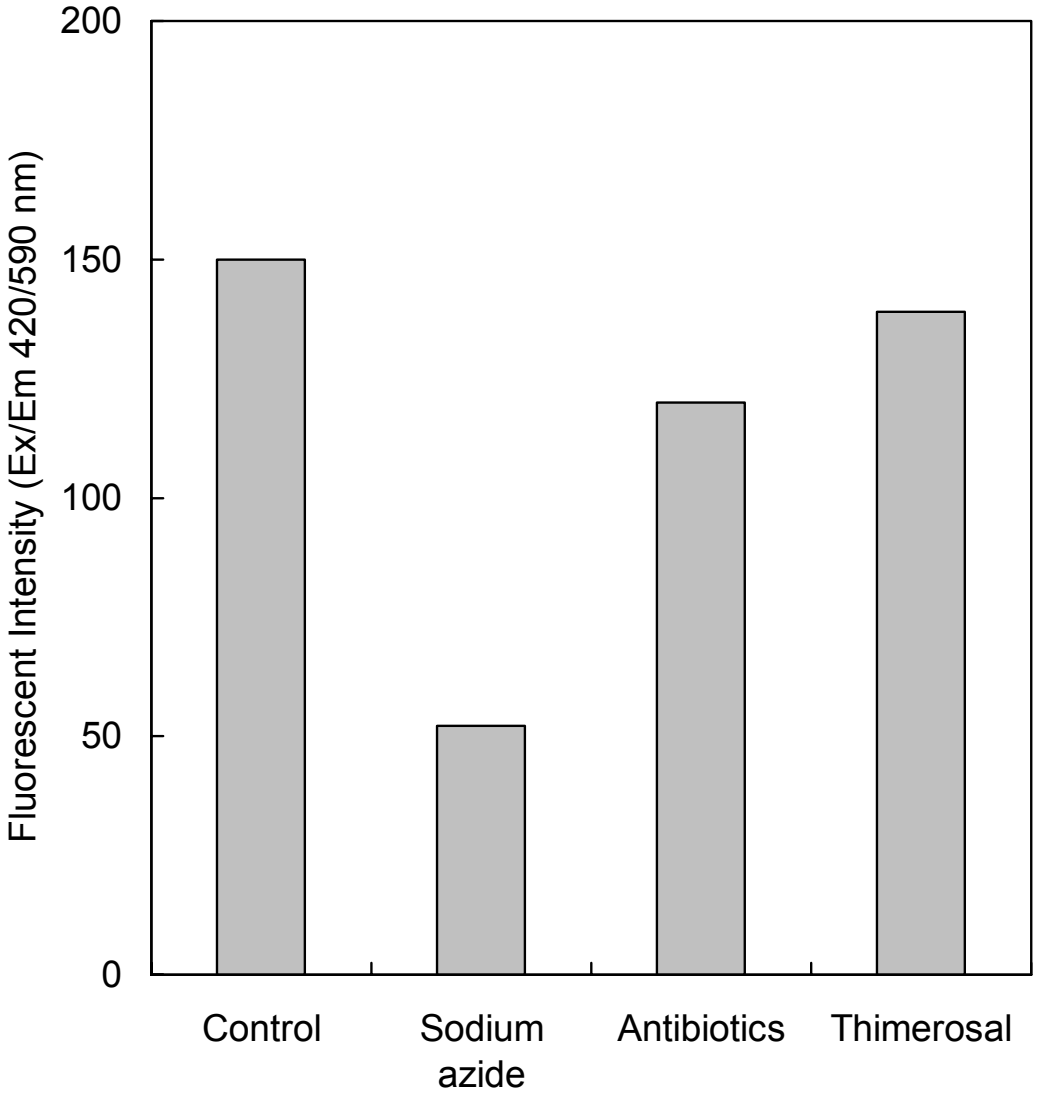

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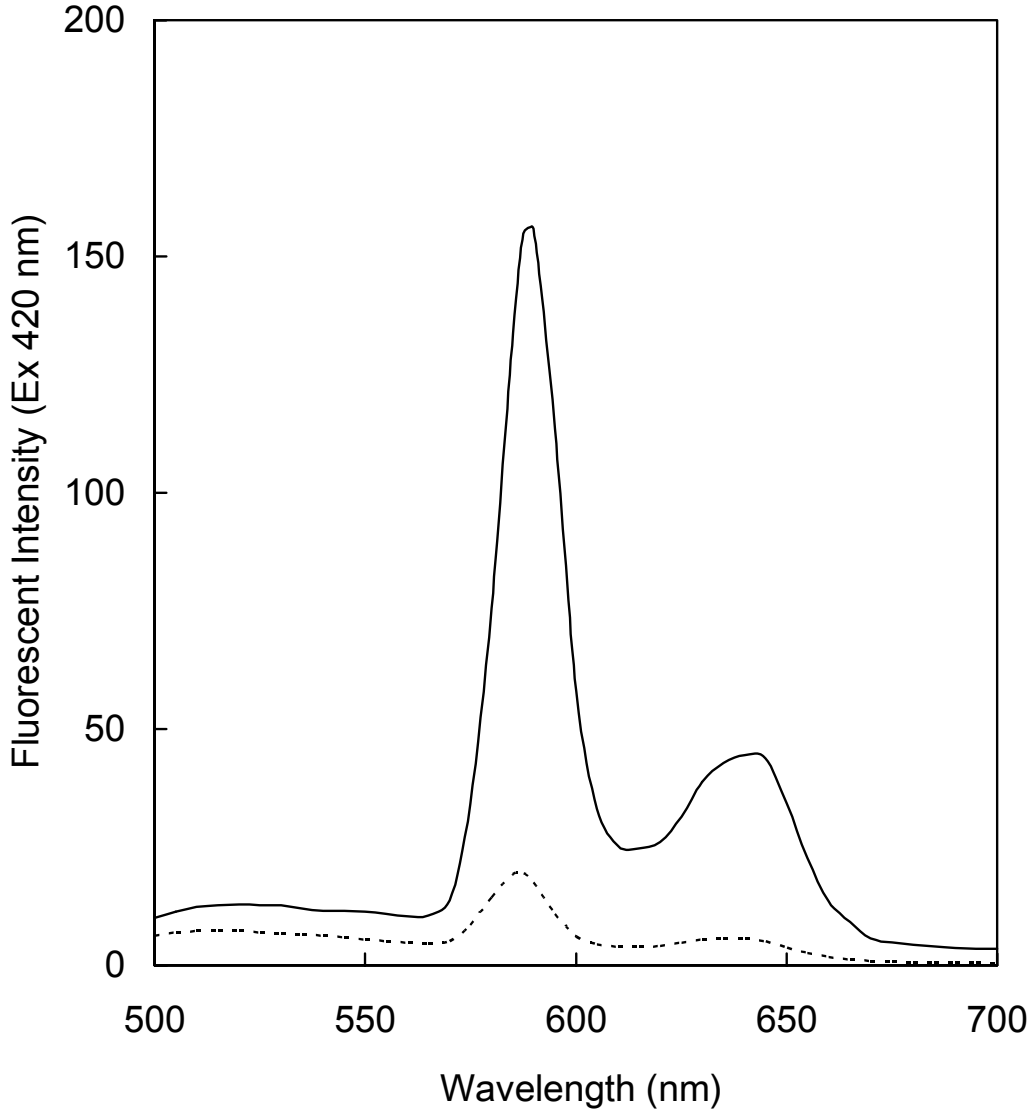

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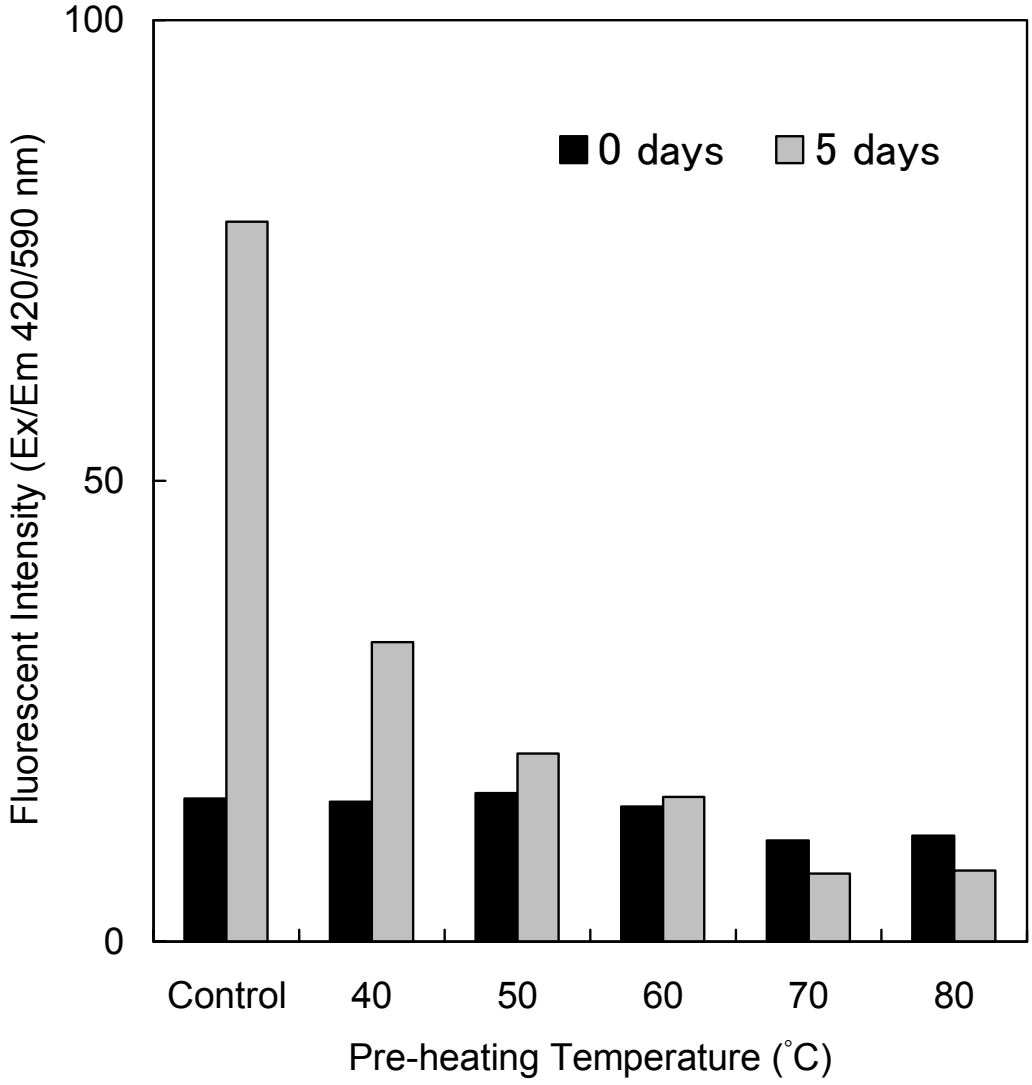

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