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Ferrochelatase Catalyzes the Formation of Zn-protoporphyrin of Dry-Cured Ham via the Conversion Reaction from Heme in Meat

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Supporting Information

ABSTRACT: Ferrochelatase (FECH), the enzyme at the last step of the heme-biosynthetic pathway, is involved in the formation of Zn-protoporphyrin via an iron-removal reaction of heme. To improve the efficacy of the formation of Zn-protoporphyrin from heme, the use of recombinant FECHs from porcine, yeast, and bacteria was examined. Incubation of FECH with myoglobin in the presence of ascorbic acid and cysteine resulted in the efficient conversion of myoglobin-heme to Zn-protoporphyrin. Exogenously added recombinant yeast FECH facilitates the production of Zn-protoporphyrin from myoglobin-heme and heme in meat, via the replacement of iron in the protoporphyrin ring by zinc ions. A large amount of Zn-protoporphyrin was also generated by the catalysis of FECH using an intact piece of meat as a substrate. These findings can open up possible approaches for the generation of a nontoxic bright pigment, Zn-protoporphyrin, to shorten the incubation time required to produce dry-cured ham.

KEYWORDS: Ferrochelatase, Zn-protoporphyrin, conversion reaction, ham pigment, dry-cured ham

INTRODUCTION

In cooked ham, the red pigment, nitrosomyoglobin, is a result of thermal treatment of meat with nitrite.[1,2] Because nitrosamines generated in nitrite-meat products are associated with a cancer risk,[2] nitrite-free or green ham has been a preferred alternative. Dry-cured ham (Parma ham), nitrite-free ham, is produced by the incubation of meat with sea salt for a long period at suitable temperature and humidity.[3] Zn-protoporphyrin as a replacement product of protoheme (Fe-protoporphyrin)[4,5] has been isolated as the main component of red pigments of these types of ham. Because of its safe and stable properties,[6−8] Zn-protoporphyrin was the preferred replacement for nitrosomyoglobin. However, it is hard to develop the red pigment rapidly in meat products, and the formation of Zn-protoporphyrin in meat is not well understood.

Ferrochelatase (FECH) (EC 4.99.1.1), located at the inner membrane of the mitochondria in mammalian cells, catalyzes the insertion of ferrous ions into protoporphyrin IX to form protoheme.[9] The gene for FECH has been isolated from various organisms, and the structures of FECH protein from bacteria to higher eukaryotes were found to be conserved. Mammalian FECH contains an iron−sulfur cluster at the carboxyl terminal of the protein,[10] whereas most fungal and bacterial FECHs do not.[5,10] The function of the cluster in the mammalian enzyme is unclear. Ferrous ions are the preferable targeting substrate of the enzyme to form heme in vivo.[9] Furthermore, other corresponding metalloporphyrins can be produced via the enzyme catalysis of divalent metal ions including zinc, cobalt and nickel with porphyrin compounds in vitro. Although it was considered that FECH irreversibly catalyzes the insertion of metal ions into porphyrin ring, we showed that FECH can also catalyze the iron-removal (reverse) reaction from heme to create protoporphyrin in vivo and in vitro and the subsequent conversion reaction from heme to Zn-protoporphyrin in vitro.[11,12] The iron-removal reaction seems to be carefully controlled in vivo,[11] but the reaction could occur in vitro. Furthermore, the reaction of NADH-cytochrome b_5 reductase (metmyoglobin reductase) reduces the ferric ions in heme to ferrous ions, which leads to the conversion of hemin to Zn-protoporphyrin.

The formation of Zn-protoporphyrin in dry-cured ham is unclear. Several findings showed that bacteria in porcine meat were the main cause of the stable pigments during Parma ham processing.[8] Recently, endogenous enzymes including FECH in meat have been identified as a potential cause of the pigment formation.[11−16] Several compounds such as salt, ascorbic acid, and dithiothreitol can increase the formation of Zn-protoporphyrin to some extent.[17−19] It is possible that the formation of Zn-protoporphyrin could occur via two pathways. First, an intermediate porphyrin, protoporphyrinogen, is oxidized by protoporphyrinogen oxidase to form protoporphyrin aerobically; then, Zn-protoporphyrin is produced by the insertion of zinc, which is present in large amounts in meat,[20] into the protoporphyrin.[21] The other pathway, also identified in our findings, is that Zn-protoporphyrin is generated via the conversion reaction of heme, in which zinc is inserted into protoporphyrin as a product of the iron-removal reaction of heme in hemoproteins catalyzed by FECH.[11,12,16] In this case, the replacement does not occur easily, especially in porcine muscle (meat). FECH from porcine muscle mitochondria catalyzes the insertion reaction of zinc into protoporphyrin to form Zn-protoporphyrin[12,13,15] as well as the iron-removal reaction of heme, strongly suggesting that FECH acts as a conversion enzyme in porcine meat. However, the mechanism of Zn-protoporphyrin in hams is still speculative.

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To obtain the highly efficient conversion from myoglobin-heme and heme in meat to Zn-protoporphyrin, we tried to use the recombinant porcine and yeast FECH for the reaction. We here demonstrate that the recombinant enzyme is effective for the production of Zn-protoporphyrin. We also found the roles of reducing agents, such as ascorbic acid and cysteine, in enhancing the iron removal and conversion from heme to Zn-protoporphyrin under anaerobic conditions. Furthermore, the addition of recombinant yeast FECH into intact piece of raw meat is applied to facilitate the generation of Zn-protoporphyrin. These results may open up new methods to generate the natural red pigment of meat and could help to shorten the incubation period of dry-cured ham.

MATERIALS AND METHODS

Materials. The genomic DNA of the thermophilic bacterium *Thermus thermophilus* (NC_006461) was obtained from Japan Gene Bank. Mesoporphyrin IX, protoporphyrin IX, and Zn-protoporphyrin were from Frontier Scientific (Logan, IT). Horse hemoglobin (Hb) and myoglobin (Mb) were products of Sigma Co. (St. Louis, MO). Porcine muscles were generously donated by Itoh Ham Inc. (Moriya, Japan). Restriction endonuclease was obtained from Takara Co. (Tokyo, Japan). The other chemicals used were of analytical grade.

Recombinant Enzymes. Mouse NADH-cytochrome b_5 reductase and porcine FECH carrying His-tag were prepared as described previously.[11,12] The FECH genes of *S. cerevisiae* and *T. thermophilus* were isolated by polymerase chain reaction (PCR) using corresponding genomic DNA. The primers used for yeast FECH were 5′-AAG GAT CCC GTC CTC ATG GCC TA-3′ (forward) and 5′-AAG AAT TCT ATC TCG GCC ACG CCG C-3′ (reverse), and those for *T. thermophilus* were 5′-AAG AAT TCG AAT GCA CAA AAG AGA T-3′ (forward) and 5′-AAA AGC TTT CAA GTA GAT TCG TGA T-3′ (reverse). The DNAs obtained were inserted into pET vector and transformed into *E. coli* BL21 strain. The bacteria were grown in LB medium for 16 h, and then, the culture medium was diluted by 10-fold in fresh LB medium. These enzymes were expressed with 0.3 mM isopropyl-β thiogalactopyranoside at 30 °C for 2 h. The cells were harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM dithiothreitol (DTT), 0.1% Tween 20, 20 mM imidazole, and 0.3 M NaCl, disrupted by sonication, and centrifuged at 500*g* at 4 °C for 10 min. The supernatants were shaken with Ni^{2+}-NTA beads (Qiagen, Valencia, CA) and washed three times with the above solution. The enzymes were eluted with 20 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1% Tween 20, 0.25 M imidazole, 1 mM DTT, and 0.3 M NaCl. The protein concentration was measured by the method of Lowry et al.[22] or Bradford,[23] using bovine serum albumin as the standard.

Electrophoresis of FECH. The proteins were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB) as described previously.[12]

Enzyme Assay. The FECH activity (forward reaction) was determined by measuring the insertion of zinc into mesoporphyrin, as described previously.[24] For measuring the iron-removal activity of FECH, a reaction mixture containing 1 mg of horse hemoglobin or myoglobin, 6 mM ascorbic acid, and 10 mM potassium phosphate buffer (pH 6.5) in a total volume of 1.0 mL was used in a Thurberg vacuum tube.[25] The dissolved gas was removed in vacuo and replaced by nitrogen. The reaction was carried out at 30 °C for 24 h. To examine the conversion of heme to Zn-protoporphyrin, 0.1 mM zinc ion was added to the reaction mixture.[11] The conversion from heme in meat to Zn-protoporphyrin was carried out as described above, except that hemoproteins were replaced by 1.0−15 g of porcine muscle (meat). Gas in the reaction tubes was removed as above and replaced with nitrogen.

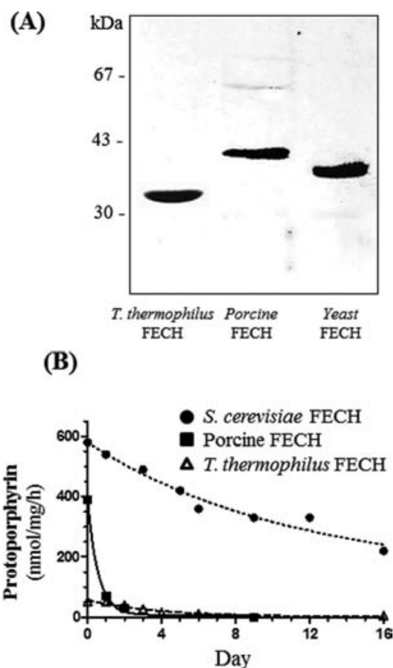

Figure 1. Characterization of the iron-removal activity and conversion reaction of porcine, yeast, and thermophilic bacterial FECH. (A) SDS-PAGE of purified porcine FECH (42 kDa), *T. thermophilus* FECH (33 kDa), and yeast FECH (39 kDa). The recombinant porcine, yeast, and thermophilic bacterial FECHs were expressed in *E. coli* and purified. One microgram of purified enzyme was analyzed by SDS-PAGE and stained using Coomassie Brilliant Blue. (B) The stability of porcine, yeast, and thermophilic bacterial FECH. The purified recombinant enzymes in 20 mM Tris-HCl (pH8.0) containing 0.3 M NaCl were stored at 18 °C for the indicated period, and the iron-removal reaction was examined. Data are expressed as means ± SDs of triplicate experiments.

Oxygen was further absorbed with a packet of AnaeroPack for Cell, disposable oxygen, and carbon dioxide-generating agent (Mitsubishi Gas Chemical Co., Tokyo, Japan). The reaction was carried out at 30 °C for 24 h.

Analysis of Metalloporphyrin and Porphyrin Pigments. Zn-protoporphyrin and protoporphyrin were extracted with acetone/ethanol (1:1 v/v) and determined by fluorospectrophotometry from 550 to 670 nm (Zn-protoporphyrin) or 600 to 700 nm (protoporphyrin) with an excitation of 400 nm (Figure S1).[12] Heme in the reaction mixture or porcine muscle was determined by the reduced−oxidized difference spectrum of pyridine hemochromogen[11] after extraction with ethyl acetate/acetic acid (3:1; v/v).

Photoimage. The extracts of the reaction mixture after incubation were transferred to quartz cuvettes and exposed to 360 nm UV light in a dark room at room temperature. A photograph of the emerged color was taken with focal ration, *f*/2.5, time of exposure, t = 1 s, and film speed, ISO = 200.

Statistical Analysis. Results were shown as means ± standard deviations (SDs) and analyzed using unpaired Student's t test. All statistical analyses were calculated significant at level of $p < 0.05$ using GraphPad Prism software version 5.02 (GraphPad Software, Inc., CA).

RESULTS

Characterization of Yeast FECH in the Conversion of Hemoproteins to Zn-protoporphyrin. We[11,12] previously reported that porcine FECH catalyzes the iron removal from heme

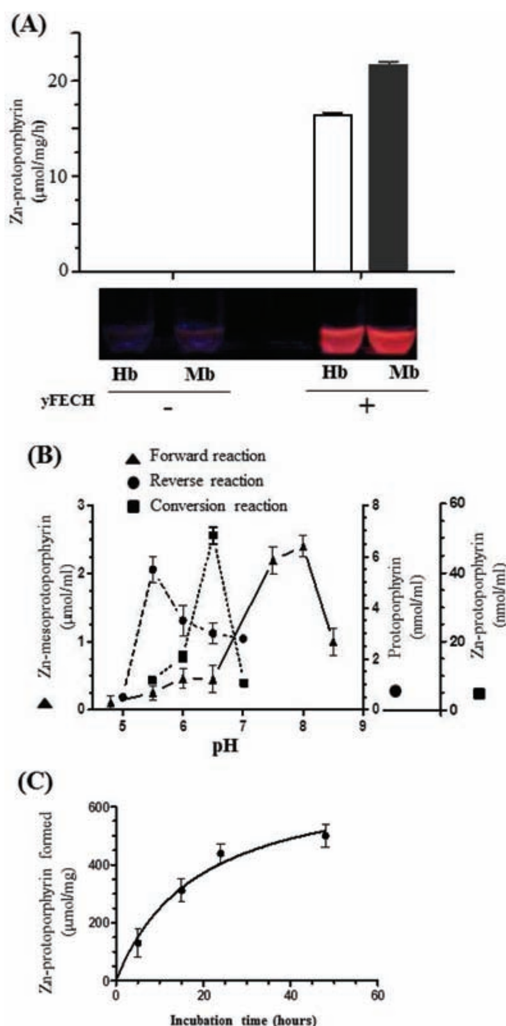

Figure 2. Kinetic study of yeast FECH. (A) The formation of Zn-protoporphyrin from hemoproteins. The reaction mixture (1.0 mL) contained 1 mg/mL myoglobin or 1 mg/mL hemoglobin, 6 mM ascorbic acid, 0.2 mM zinc acetate, and 0.3 μg of yeast FECH, in 10 mM potassium phosphate buffer, pH 6.5. The reaction was carried out at 30 °C for 24 h, and the formation of Zn-protoporphyrin was measured (upper panel). The ethanol/acetone extracts in the cuvettes were exposed to UV light in a dark room. The fluorescent image after the reaction was observed (lower panel), and the red color shows the production of Zn-protoporphyrin. (B) Effect of pH. The reactions were performed under the conditions as described above, except for the use of 10 mM potassium phosphate buffer with indicated pH. (C) Time course on the formation of Zn-protoporphyrin from myoglobin. The reactions were performed as described above, except that the incubation period was varied. Data are expressed as means ± SDs of 2−4 independent experiments.

as well as the conversion of heme to Zn-protoporphyrin and suggested that a large amount of Zn-protoporphyrin in Parma ham may be generated via iron-removal and conversion reactions of FECH from myoglobin-heme. However, the reaction was not fully identified, and the porcine enzyme only produced a small amount of Zn-protoporphyrin from hemin.[12] Thus, we tried to improve conditions to obtain a high yield of Zn-protoporphyrin from myoglobin by FECH. First, we searched for FECH from other sources because the reverse and conversion activities using mammalian FECH were not high because of instability of mammalian FECH. We cloned FECH genes from the bacterium *T. thermophilus* and the yeast *S. cerevisiae* and expressed them in *E. coli.* The recombinant enzymes were purified using a nickel column and analyzed by 10% SDS-PAGE. The molecular masses of porcine, yeast, and bacterial FECH were 42, 39, and 33 kDa, respectively (Figure 1A). We then compared the iron removal (reverse activity) and stability of the enzymes (Figure 1B). When the enzymes were freshly prepared, the strongest activity was obtained with yeast enzyme. Porcine enzyme showed two-thirds of the activity of the yeast enzyme, and the bacterial one showed the lowest activity. To compare the stability of the three enzymes, they in 20 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl were stored at 18 °C for the indicated period, and then, the reverse activity was examined. Yeast FECH showed 42% of the initial activity after storage for half a month, corresponding to a half-life of the activity ($t_{0.5}$) of 12 days, whereas porcine FECH was unstable and showed $t_{0.5}$ of 12 h. When the yeast enzyme was stored at 4 °C for a month, virtually no loss of the activity was observed. The bacterial FECH showed lower activity although it was more stable than the porcine enzyme. Thus, the yeast FECH showed strong activity and was suitable to obtain a high yield of Zn-protoporphyrin. Figure 2A shows the formation of Zn-protoporphyrin with myoglobin and hemoglobin catalyzed by yeast FECH. A considerable amount of Zn-protoporphyrin was formed with 1 mg/mL myoglobin or hemoglobin, whereas no Zn-protoporphyrin was found in the reactions without FECH. After Zn-protoporphyrin in the extracts was determined, the extracts upon exposure to UV light showed strong red fluorescence (Figure 2A, lower panel). Thus, yeast FECH catalyzes the conversion reaction from hemoproteins to Zn-protoporphyrin. Although the forward reaction of FECH showed an optimum at pH 7.5−8.0, the reverse one did at pH 5.5 (Figure 2B), which was consistent with previous observations.[12] When the pH profile of the conversion reaction from hemoglobin to Zn-protoporphyrin was examined, the highest activity was obtained at pH 6.5.

Then, we examined the time course of the conversion reaction using myoglobin as a substrate. To protect the stability of FECH and hemoproteins, we selected the incubation temperature of 30 °C. The formation of Zn-protoporphyrin gradually increased with time and reached a plateau by 24 h of incubation (Figure 2C). Kinetic properties of the conversion reaction from myoglobin and Zn^{2+} were also determined. The enzyme showed a V_{max} value of about 30 μmol of Zn-protoporphyrin formed/mg of protein/h, and K_m values for Zn^{2+} and myoglobin were 60 (Figure S2A in the Supporting Information) and 12 μM (Figure S2B in the Supporting Information), respectively.

To remove iron from heme, the reduced heme in myoglobin is required.[11] Thus, we examined the effect of reductants on the promotion of the reaction. NADH-cytochrome b_5 reductase can maintain the reduced form of myoglobin. The addition of recombinant NADH-cytochrome b_5 reductase with NADH caused weak conversion activity. Ascorbic acid (6 mM) and cysteine (6 mM) as additives showed marked stimulation (Figure S3 in the Supporting Information). Glutathione (6 mM) showed a weak effect (data not shown). When the concentration of ascorbic acid was changed, the activity increased dependent on the concentration up to 6 mM and then decreased (Figure 3A). Cysteine at a high concentration was effective. It is known that pig leg is treated with salt powder at the first step of dry-cured ham processing.[3] We thus examined whether NaCl affects the formation of Zn-protoporphyrin from myoglobin-heme. As

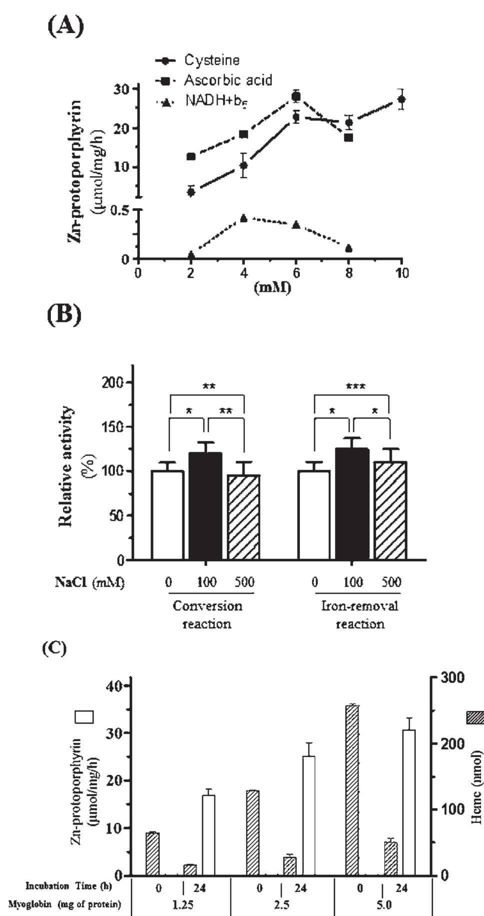

Figure 3. Formation of Zn-protoporphyrin of recombinant yeast FECH with myoglobin-heme or hemoglobin-heme. (A) Effect of reductants with yeast FECH on the conversion of myoglobin-heme to Zn-protoporphyrin. Yeast FECH was incubated in the reaction mixture similar to that described in the legend to Figure 2, except that the concentration of reductants was varied. NADH (2−8 mM) and mouse NADH-cytochrome b_5 reductase (10 μg of protein) for replacement of reductants were also used. (B) Effect of NaCl. The reaction was performed under the conditions as described above, except for the addition of the indicated concentration of NaCl. Data were tested the significant by using Student's t test, $0.05 < {}^{*}p < 0.1$, $0.1 < {}^{**}p < 0.5$, and ${}^{***}p > 0.5$. (C) Change in the proportion of Zn-protoporphyrin and myoglobin-heme by yeast FECH. The conversion reaction was performed similarly to the conditions as described above, except for the use of the indicated concentration of myoglobin. Protoporphyrin and Zn-protoporphyrin were examined by fluorospectrophotometry. Heme was determined using the reduced-oxidized pyridine hemochromogen. Data are expressed as means ± SDs of triplicate experiments.

shown in Figure 3B, the addition of NaCl at all concentrations did not show any significant statistical effect.

We next examined the change in the proportion of Zn-protoporphyrin to heme by the enzyme reaction. The amount of Zn-protoporphyrin increased after the incubation, which was accompanied by the decrease of heme (Figure 3C). At all examined concentrations of myoglobin, Zn-protoporphyrin was produced in a manner dependent on the initial concentration of myoglobin. It is evident that the conversion reaction of Zn-protoporphyrin from myoglobin-heme occurs via the catalysis of FECH.

Effect of Yeast FECH on the Conversion of Heme in Porcine Muscle (Meat) to Zn-protoporphyrin. We examined the effect of FECH on the formation of Zn-protoporphyrin from heme in porcine muscle (meat). Meat (1 g) was added to the reaction mixture containing 6 mM ascorbic acid and porcine FECH or yeast FECH (0.3 μg). After anaerobic incubation at 4 or 30 °C for 24 h, the reaction mixtures with meat were homogenized, and porphyrins were extracted with ethanol/acetone (1:1, v/v). The results in Figure 4A (upper panel) revealed that the formation of Zn-protoporphyrin without recombinant FECH occurred at 30 °C. The yield of Zn-protoporphyrin with yeast enzyme was much higher than those without or with exogenous porcine FECH. The pink color corresponding to Zn-protoporphyrin by UV light was observed in the case of 30 °C incubation, and the intensity with recombinant FECH was stronger than that without FECH (lower panel). Using meat as a reaction source, the addition of zinc acetate to

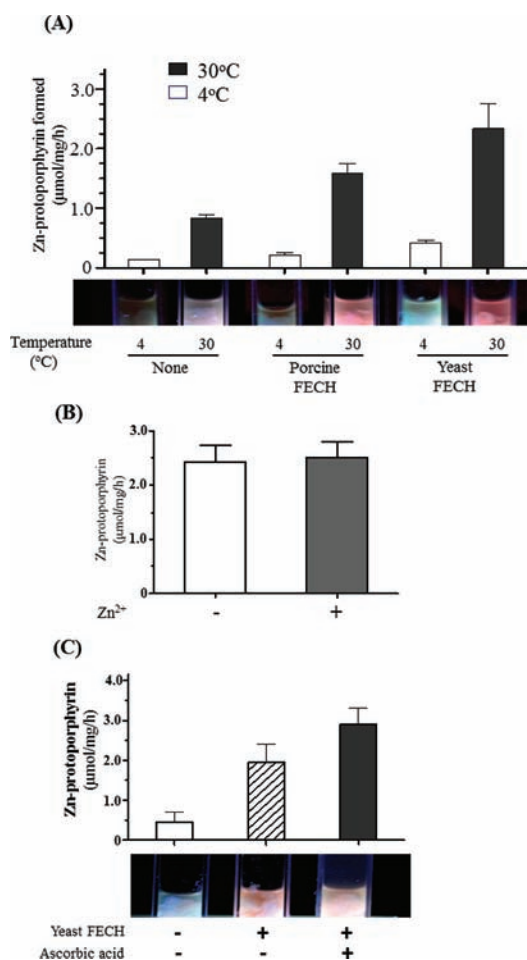

Figure 4. Conversion of heme in porcine muscle to Zn-protoporphyrin. (A) The reaction mixture containing porcine muscle (1 g wet weight) and 10 mM potassium phosphate buffer, pH 6.5, in the presence of porcine and yeast FECH (0.3 μg) was incubated at 4 or 30 °C for 24 h. The formation of Zn-protoporphyrin was measured (upper panel). Data are expressed as means ± SDs of triplicate experiments. The fluorescence in the ethanol/acetone extracts was observed (lower panel). (B) Effect of exogenous zinc ions on the conversion reaction. The reaction was performed under the conditions as described above, except for the addition of 0.2 mM zinc acetate. (C) Effect of ascorbic acid (upper panel). The incubation as above was carried out with or without 6 mM ascorbic acid. The lower panel shows a fluorescent image of the Zn-protoporphyrin (pink) produced.

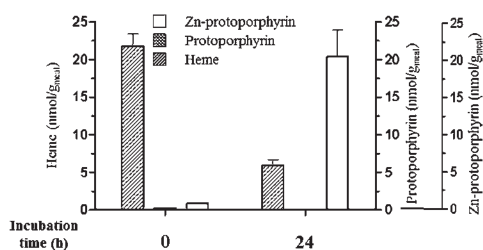

Figure 5. Proportion of metalloporphyrins and protoporphyrin in porcine muscle by the incubation with yeast FECH. The reaction mixture containing 1 g of meat (porcine muscle), 6 mM ascorbic acid, 0.3 μg of yeast FECH, and 10 mM potassium phosphate buffer (pH 6.5) was incubated at 30 °C for 24 h. The contents of Zn-protoporphyrin, protoporphyrin, and heme were examined. Data are expressed as means ± SDs of 2−4 independent experiments.

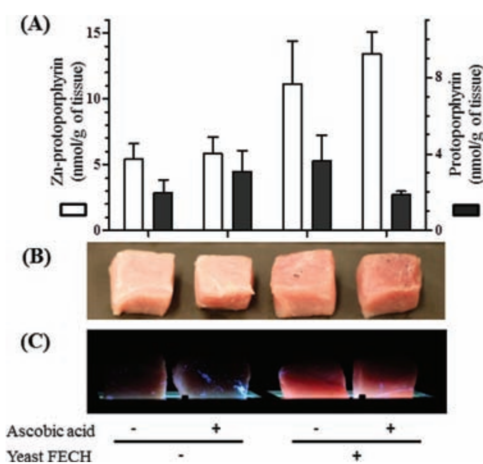

Figure 6. Conversion of heme in meat to Zn-protoporphyrin using the intact piece of porcine muscle. (A) The formation of Zn-protoporphyrin from heme in tissues of porcine muscle. The solution (1.0 mL) containing 1 μg of yeast FECH, 6 mM ascorbic acid, and 10 mM potassium phosphate buffer, pH 6.5, was injected into porcine muscle (15 g). The meat was incubated anaerobically at 30 °C for 24 h. After incubation, Zn-protoporphyrin and protoporphyrin were extracted from meat, using acetone/ethanol (1:1 v/v), and determined fluorophotometrically. Data are expressed as means ± SDs of triplicate experiments. Intact pieces of porcine muscle after incubation were exposed to white light (B) or UV light in a dark room (C), and then, the images were observed. The pink color indicates the production of Zn-protoporphyrin.

the mixture did not change in the formation of Zn-protoporphyrin, indicating that zinc ions are present at sufficiently high levels in meat[20] for the zinc ion-insertion reaction to proceed (Figure 4B). When meat as a substrate material was used, a considerable amount of Zn-protoporphyrin was formed without ascorbic acid, and the addition of ascorbic acid (6 mM) showed enhancement on the formation (Figure 4C). Figure 5 shows the change in the composition of heme and Zn-protoporphyrin in meat by the incubation with yeast FECH. At the initial time, heme was the main component of porcine muscle, but the amount of Zn-protoporphyrin increased sharply to 73% of the initial content of heme, whereas heme was decreased to 27%. These results indicated that the addition of the recombinant yeast FECH with ascorbic acid effectively enhanced the conversion reaction from heme in meat to Zn-protoporphyrin.

Formation of Zn-protoporphyrin from Heme in the Intact Piece of Porcine Muscle. The above results demonstrated that FECH is effective for the formation of Zn-protoporphyrin using a small amount of meat with the liquid reaction mixture. We finally examined the conversion using the intact piece of meat (15 g) as a reaction source. The solution (1 mL) containing yeast FECH (1 μg) and 6 mM ascorbic acid was injected into muscle, and the treated meat was then incubated anaerobically. As shown in Figure 6A, the formation of Zn-protoporphyrin in the muscle was observed after 24 h of incubation at 30 °C. Zn-protoporphyrin was formed in samples either with or without ascorbic acid. In addition, a considerable amount of protoporphyrin in meat was also formed. The photoimages indicated a bright red color of meat that emerged upon direct exposure of meat to both visible and UV light (Figure 6B,C). This proves that yeast FECH acts as a promoter of the conversion reaction to Zn-protoporphyrin from heme in meat.

DISCUSSION

The present study first demonstrated that highly efficient iron removal and subsequent conversion of heme in meat to Zn-protoporphyrin occur upon a relatively short period of incubation, via the catalysis of FECH. The replacement of iron by zinc in the formation of Zn-protoporphyrin was noted previously.[15−17] Our previous studies[11,12] showed that the formation of Zn-protoporphyrin can take place via the reverse and conversion reactions and was catalyzed by FECH. However, the level of production of Zn-protoporphyrin from heme is low because the reaction of FECH precisely proceeds to the metal ion-insertion (forward) reaction. We also found that a considerable concentration of reducing reagents including ascorbic acid and cysteine under anaerobic conditions was required for the highly efficient production of Zn-protoporphyrin. In addition, the use of recombinant FECH enhanced the conversion reaction of heme, with hemin, hemoglobin-heme, and myoglobin-heme as well as heme in meat strongly suggesting that FECH can catalyze the conversion reaction from any heme in tissues.

The formation of Zn-protoporphyrin without the decrease of heme was previously noted using exogenous myoglobin or meat extracts,[21] indicating that the formation could follow the other pathway independent of FECH catalysis. On the other hand, we previously[11] reported that 67% of pigment of dry-cured ham was Zn-protoporphyrin, only 10% was heme, and the rest was protoporphyrin. The present study demonstrated that about 70% of metalloporphyrin in meat was Zn-protoporphyrin, which was accompanied by the decrease of heme after 24 h of incubation with yeast FECH at 30 °C (Figure 5). These results confirmed that endogenous porcine FECH in meat is responsible for the formation of Zn-protoporphyrin, and the formation of Zn-protoporphyrin is dependent upon the initial heme concentration in dry-cured ham including Parma ham.

We previously reported that the iron-removal activity of heme with mouse FECH upon a short period of incubation had an optimum temperature of 45 °C.[12,19] Thus, we tried to use FECH of thermophilic bacteria for the formation of Zn-protoporphyrin because enzymes in these bacteria are relatively stable at high temperature. We expressed the recombinant FECH of *T. thermophilus* in *E. coli* and purified it. When the conversion of heme to Zn-protoporphyrin with *T. thermophilus* FECH was examined at 45 °C, the activity was unexpectedly low (data not shown). The bacterial enzyme activity was about 10% of that of the porcine

enzyme (Figure 1B). *E. coli* FECH was also examined for the conversion activity but was ineffective on the activity (data not shown). Among the enzymes examined, yeast FECH showed the highest activity.

It is known that mammalian FECH includes an iron−sulfur cluster at the carboxyl terminal. At present, the roles of the cluster are not fully understood. This cluster could play an important role in mammalian FECH activity.[26,27] The cluster can also be found in FECH from some kinds of bacteria and yeast *Schizosaccharomyces pombe*,[28] but its role is not clear. We compared the stability of the cluster-free FECH of the bacterium *T. thermophilus*, the yeast *S. cerevisiae*, and the porcine enzyme containing the cluster. Although the iron−sulfur cluster could not be related to the ability of the reverse and conversion reactions of FECH, the differences in stability of bacterial, mammalian, and yeast FECH could be related to the presence of the cluster. Oxygen, nitric oxide, and various chemicals can easily destroy the iron−sulfur cluster.[26] This was supported by the observations that the formation of Zn-protoporphyrin was decreased by the addition of nitrite in the processing of dry-cured ham.[17,21] Therefore, the mammalian FECH was unstable as compared with those of yeast and bacteria. The yeast FECH was quite stable with high activity (Figure 1B). Thus, yeast FECH can be the model supplement enzyme to obtain a high yield in the conversion of Zn-protoporphyrin from heme.

Ascorbic acid has been used as a preserving additive in meat products.[29] As shown previously, reducing systems can play a vital role in the reverse and conversion reactions of FECH. NADH-cytochrome b_5 reductase (metmyoglobin reductase) can reduce heme to enhance the reverse reaction.[11] In vitro, ascorbic acid and cysteine showed the same effect as NADH-cytochrome b_5 reductase on the reduction of ferric ions to ferrous ions, and then, the FECH can attack to remove ferrous ions in heme. In addition, ascorbic acid as well as cysteine at 6 mM can highly promote the reverse and conversion activities of FECH (Figure 3A). The enhancing ability of ascorbic acid on the formation of Zn-protoporphyrin found in this study is in agreement with another finding[18] that ascorbic acid can promote the formation of the pigments of dry-cured ham. The decrease of FECH activity at a higher concentration of ascorbic acid can be explained by the decrease of pH value. On the other hand, the formation of Zn-protoporphyrin in meat via the conversion reaction occurred without any addition of exogenous reductants, showing that some reducing systems in meat are present at a significant level or can be derived from some kinds of bacteria. The endogenous reductants such as ascorbic acid, glutathione, and nicotine nucleotides in meat can help the endogenous FECH-dependent occurrence of iron-removal and the zinc ion-insertion reactions of heme in meat during the processing of ham.

Other investigators[17,18] reported that the treatment of meat with NaCl improved the formation of Zn-protoporphyrin in dry-cured ham. A similar ability of salts was also found in the reaction system using meat extracts as FECH sources.[18] In these studies, because zinc ions and protoporphyrin were added to the reaction mixture, the formation of Zn-protoporphyrin could only occur via the forward reaction. In contrast, the present study showed that NaCl did not have any effects on the formation of Zn-protoporphyrin via the reverse and conversion reactions from hemoproteins catalyzed by FECH. The reason for this difference is unclear, but it is possible that the enhancement of the zinc-insertion reaction by NaCl contributed to different enzyme sources and experimental conditions. The other possibility is that the NaCl in ham maintains suitable growing conditions for yeast and some kinds of bacteria, which can enhance the formation of Zn-protoporphyrin in dry-cured ham.[8] However, the formation of Zn-protoporphyrin slightly decreased in the antibiotic-treated samples,[14] indicating that bacteria showed minor roles in the conversion reaction of Zn-protoporphyrin in dry-cured ham processing. Otherwise, it is possible that the addition of NaCl to meat during ham processing can prevent the growth of spoiling bacteria.[3]

Zinc ions can compete with iron in the insertion of divalent metal ions to protoporphyrin to form the corresponding metalloporphyrin.[30] In the case of reverse and conversion reactions of heme, the amount of protoporphyrin is lower than that of Zn-protoporphyrin in the same reaction condition, indicating that zinc ions can enhance the removal reaction to remove the protoporphyrin, a substrate of the forward reaction. When porcine meat was used, the high level of conversion of heme to Zn-protoporphyrin occurred without the addition of exogenous zinc ions, indicating that zinc ions are abundant in meat[20] and are present at sufficient levels for the conversion reaction of heme to Zn-protoporphyrin. The amount of Zn-protoporphyrin formed in meat by incubation at 4 °C was less than that by incubation at 30 °C. This result agreed with the findings that the level of Zn-protoporphyrin did not increase considerably at low temperature during the incubation and production of dry-cured ham, and it just increased during the midtemperature incubation stage of the processing.[13]

It was reported that meat extracts acted as the enzyme sources for Zn-protoporphyrin formation,[13,16,18] and the iron-removal and conversion reactions of myoglobin to Zn-protoporphyrin were successfully demonstrated.[15−17] Although exogenous protoporphyrin, myoglobin, and zinc ions were added as substrates for these reaction mixtures, the yield in the formation was low. The present data showed that not only porcine FECH in porcine muscle (raw meat) but also yeast FECH as exogenously added enzyme used the endogenous myoglobin-heme in meat as a substrate, promoting the iron-removal and conversion reactions of heme to Zn-protoporphyrin.

The oxidation of protoporphyrinogen to protoporphyrin catalyzed by protoporphyrinogen oxidase occurs in vivo in the heme-biosynthetic pathway,[31] and this protoporphyrin can be utilized for the formation of Zn-protoporphyrin. However, the sustained activity of the enzyme in meat in vitro has not been demonstrated yet, or the enzyme is unstable.[32] Because the enzyme can be destroyed easily after cell death, this oxidation process probably did not occur during the processing of dry-cured ham, indicating that the insertion of zinc into protoporphyrin after the oxidation of protoporphyrinogen cannot regularly occur. Therefore, the replacement of iron by zinc ions occurs via the reverse, and conversion reactions of heme in meat cause the formation of Zn-protoporphyrin.

The present data on the conversion reaction from heme to Zn-protoporphyrin revealed that yeast recombinant FECH can shorten the period of formation of ham pigments with a high yield. The sensory quality of dry-cured ham consists of color, flavor, and texture. Flavor involves nonvolatile (taste) and volatile compounds (aroma) including free amino acids, peptides, fatty acids, and other natural organic compounds; texture relates to myofibrillar protein breakdown, extent of drying, degradation of connective tissues, and the intramuscular fat.[3] The positive changes of flavor and texture properties can be developed up to 1−2 years of maturation and relate to the

proteolysis and lipolysis[3] in which FECH could not be involved.

The conversion of hemoporotein-heme to Zn-protoporphyrin by FECH showed an optimum at pH 6.5 (Figure 2B), whereas pH in raw meats is 5.5−6.0.[6,7] On the basis of the observations that the formation of Zn-protoporphyrin from myoglobin-heme readily proceeds in the raw tissues (Figures 5 and 6), some additional factors may be involved in the enhancement of the formation in meat. Yeast FECH showed the high conversion and iron removal activities at high NaCl concentration (up to 500 μM) (Figure 3B). This demonstrates that the enzyme can be applied to dry-cured ham production, by the addition of 20−30 g NaCl/kg raw meat,[3] in some stages to generate only the pigments, Zn-protoporphyrin, or protoporphyrin, of the ham.

Further studies will be carried out on the application of FECH to the dry-cured ham processing or that of other meat products to find suitable conditions for the enzyme reaction. Other studies should examine the effect of halophilic bacteria that can produce superior FECH or redox enzymes that are effective for the generation of ham pigments.

■ ASSOCIATED CONTENT

Supporting Information. Figures of fluorescent profiles of Zn-protoporphyrin and protoporphyrin, effect of substrate concentrations on the conversion activity, and effect of reductants on the conversion activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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