

**Egg white hydrolysate inhibits oxidation in mayonnaise and a model system**

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5 **Title:** Egg white hydrolysate inhibits oxidation in mayonnaise  
6 and a model system

7

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25

26 *Abbreviations:* DMSO, dimethyl sulfoxide; DPPH,  
27 1,1-diphenyl-2-picrylhydrazyl; EWH, egg white hydrolysate;  
28 EDTA, ethylenediaminetetraacetic acid; PV, peroxide value; TCA,  
29 trichloroacetic acid.

1 **Abstract**

2           The flavor deterioration of mayonnaise is induced by iron,  
3 which is released from egg yolk phosphovitin under acidic  
4 conditions and promotes lipid oxidation. To prevent oxidative  
5 deterioration, natural components, rather than synthetic  
6 chemicals such as ethylenediaminetetraacetic acid have been  
7 required by consumers. In the present study, we evaluated the  
8 inhibitory effects of three egg white components with the same  
9 amino acid composition, namely egg white protein, hydrolysate,  
10 and the amino acid mixture, on lipid oxidation in mayonnaise and  
11 an acidic egg yolk solution as a model system. We found that the  
12 hydrolysate had the strongest inhibitory effect on lipid oxidation  
13 among the three components. The mechanism underlying the  
14 antioxidant effect was associated with Fe<sup>2+</sup>-chelating activity.  
15 Thus, egg white hydrolysate may have the potential as natural  
16 inhibitors of lipid oxidation in mayonnaise.

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19 **Key words:** chelate, egg white, hydrolysate, mayonnaise,  
20 oxidation

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1        Mayonnaise is an acidic oil-in-water emulsion food that  
2 consists of vegetable oil, eggs, and vinegar. It contains nutrients  
3 such as polyunsaturated fatty acids and iron derived from egg  
4 yolk phosvitin,<sup>1)</sup> which contains many phosphorylated serine  
5 residues.<sup>2)</sup> Iron, which is released from egg yolk phosvitin  
6 because of the decrease in pH caused by acetic acid in vinegar,  
7 induces the oxidative degradation of mayonnaise.<sup>3)</sup> In other  
8 words, phosvitin has little chelating activity in mayonnaise. To  
9 maintain the commercial value of mayonnaise, calcium disodium  
10 ethylenediaminetetraacetic acid (EDTA) has been used as a food  
11 additive over a long period in most countries. EDTA is an  
12 economical agent that is highly effective in strongly chelating  
13 iron. However, EDTA is also a chemical synthetic product that  
14 tends to give a negative image to consumers. Thus, antioxidants  
15 derived from a natural product are required as follows.<sup>4-8)</sup>  
16 Previously, it was evaluated whether natural antioxidants such as  
17 phytic acid,<sup>4)</sup> gallic acid,<sup>5)</sup> tocopherol,<sup>6)</sup> ascorbic acid,<sup>7)</sup> and  
18 purple corn husk extracts<sup>8)</sup> inhibited the oxidation of lipids in  
19 mayonnaise. Among these components, only purple corn husk  
20 extracts inhibited lipid oxidation in mayonnaise, but the color of  
21 mayonnaise turned purple. We have a concern that the purple  
22 color will not be acceptable to consumers.

23        In addition to these natural antioxidants, some proteins and  
24 peptides are also known to function as antioxidants.<sup>9)</sup> Egg  
25 albumin inhibited the iron-catalyzed oxidation of egg  
26 phosphatidylcholine.<sup>10)</sup> Fat-free egg yolk protein exerted an  
27 antioxidant effect on ethanol/water or on cookies containing  
28 linoleic acid. Compared with egg-yolk protein, the hydrolysate  
29 exhibited a stronger antioxidant effect.<sup>11)</sup> Because the color of

1 these egg proteins/hydrolysate is white, the egg-derived  
2 components may prevent the oxidation of lipids in foods without  
3 changing the color of mayonnaise.

4 As described above, acetic acid caused the release of iron,  
5 subsequently leading to lipid oxidation. In addition, acetic acid  
6 itself may directly participate in lipid oxidation in mayonnaise  
7 because acetic acid was reported to accelerate the oxidation of  
8 soybean oil.<sup>12)</sup> On the other hand, some organic acids have an  
9 antioxidative effect,<sup>12-14)</sup> and they may inhibit the lipid  
10 oxidation in mayonnaise.

11 Hence, in the present study, we determined the inhibitory  
12 effect of egg white components and organic acids, instead of  
13 acetic acid, on lipid oxidation using real mayonnaise and a model  
14 system, and the antioxidative mechanisms were also evaluated.

15

## 16 **Materials and Methods**

17 *Materials.* Shelled eggs were purchased from a  
18 supermarket in Tokyo. Glacial acetic acid of food additive grade  
19 was purchased from The Nippon Synthetic Chemical Industry Co.,  
20 Ltd. (Osaka, Japan). Calcium disodium EDTA was purchased  
21 from Maruzen Chemicals Co., Ltd. (Tokyo, Japan). Trolox and  
22 neocuproine were purchased from Sigma-Aldrich (St Louis, MO,  
23 USA). Egg white protein hydrolysate EP-1® is a product of  
24 Kewpie Co. (Tokyo, Japan). It is obtained by treating albumin  
25 with neutral protease of *Aspergillus* origin. Its average  
26 molecular weight is 1100. Citric acid,  
27 1,1-diphenyl-2-picrylhydrazyl (DPPH), and  
28 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid  
29 monosodium salt hydrate (ferrozine) were purchased from Wako

1 Pure Chemical Industries, Ltd. (Osaka, Japan). Ammonium iron  
2 (II) sulfate hexahydrate,  $\text{FeCl}_3$ , 100% trichloroacetic acid (TCA),  
3 and 5-sulfosalicylic acid dihydrate were purchased from Nacalai  
4 Tesque, Inc., (Kyoto, Japan). EDTA-2Na was purchased from  
5 Dojindo Laboratories (Kumamoto, Japan). All other chemicals  
6 were of analytical reagent grade.

7

8 *The amino acid compositions of the egg white protein and*  
9 *hydrolysate, and preparation of the amino acid mixture.*

10 Egg white was freeze-dried and then pounded in a mortar. The  
11 resulting compound was used as egg white protein. The amino  
12 acid compositions of egg white protein and EP-1 were measured  
13 using a JLC/500V2 amino acid analyzer (Nippon Denshi, Tokyo,  
14 Japan) as follows. Egg white protein and EP-1 were hydrolyzed  
15 by HCl. Cysteine was oxidized to cysteic acid using performic  
16 acid prior to hydrolysis.<sup>15)</sup> Tryptophan content was determined  
17 via barium hydroxide hydrolysis.<sup>16)</sup> The amino acid contents of  
18 egg white protein and EP-1 are shown in Table 1. The findings  
19 were similar between the two.

20 To prepare an amino acid mixture that has the same  
21 formulation ratio as EP-1, 18 amino acids were purchased from  
22 Kanto Chemical Co., Inc. (Tokyo, Japan) and were then  
23 compounded.

24

25 *Preparation of acidic egg yolk solution as a model system for*  
26 *lipid oxidation in mayonnaise.* The solid content of egg  
27 yolk was adjusted to 43% via the addition of a small amount of  
28 egg white, according to the literature.<sup>17)</sup> After the egg yolk  
29 mixture (10 g) was diluted with distilled water (130 g), the

1 diluted egg yolk solution was heated at 60°C for 3 min. Further,  
2 the egg yolk solution was adjusted to pH 4.0 using organic acids  
3 such as glacial acetic acid. The final solid content of egg yolk in  
4 the acidic egg yolk solution was 1%. Egg white components as  
5 candidate antioxidants were dispersed with a vortex mixer  
6 (model TM-151; AGC Techno Glass Co., Ltd. Shizuoka, Japan) in  
7 the distilled water. These components were added to the acidic  
8 egg yolk solution, and then the antioxidant action was evaluated  
9 as follows.

10 The acidic egg yolk solution (10 mL) in a 15-mL glass tube of  
11 11 mm inner diameter with a screw cap was incubated at 55°C in  
12 the dark. Lipid oxidation was evaluated by measuring the  
13 fluorescence intensity according to previous reports.<sup>18-20)</sup> In  
14 brief, ether/ethanol (1:3, v/v) was added to an aliquot of the  
15 oxidized reaction mixture and then centrifuged at 1,200 g for 5  
16 min at room temperature. The upper phase was measured at  
17 excitation and emission wavelengths of 360 and 440 nm,  
18 respectively, using a Hitachi F-2000 spectrophotofluorometer.  
19 The fluorescence intensity was expressed as a value relative to  
20 the standard value for 1 µg/mL quinine sulfate/0.1N sulfuric acid  
21 set as 100.

22

23 *GC-MS analysis.* Volatile compounds produced by lipid  
24 oxidation from the acidic egg yolk solution were analyzed by  
25 GC-MS according to a previous report.<sup>21)</sup> In brief, the acidic egg  
26 yolk solution (3 mL) was incubated in a sealed glass tube at 40°C  
27 for 5 min. Volatile compounds were adsorbed via headspace solid  
28 phase microextraction using a  
29 polydimethylsiloxane/Carboxen/divinylbenzene (Sigma-Aldrich)

1 fiber at 40°C for 20 min and then analyzed by GC using an  
2 Agilent 6890 gas chromatograph coupled to a 5973 mass  
3 spectrometer (Agilent Technologies, Palo Alto, CA) with  
4 electron impact ionization at 70 eV. Mass units were monitored  
5 from 29 to 290 m/z. Separation was performed on a Sol-Gel-Wax  
6 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness;  
7 SGE Analytical Science, Victoria, Australia) carrying a constant  
8 flow of helium (1.0 mL/min). The inlet temperature was set at  
9 250°C. The column oven temperature program consisted of an  
10 initial condition of 35°C held for 5 min, followed by an increase  
11 to 120°C at a rate of 5°C/min and then 220°C at a rate of  
12 15°C/min, followed by a final hold time of 6 min. The mass  
13 spectra of peaks were identified by comparison with the NIST  
14 database mass spectral library.

15  
16 *Preparation of mayonnaise.* Materials for mayonnaise  
17 were compounded using the formulation shown in Table 2 and  
18 then passed through the colloid mill of a Kewpie pilot plant  
19 (Kewpie Co.) at room temperature. Thereby, mayonnaise  
20 formulations having an average particle diameter of 2–5 μm were  
21 obtained. The particle diameter was determined using a laser  
22 diffraction particle size analyzer (model SALD-200V; Shimadzu,  
23 Kyoto, Japan). The mayonnaise formulations (200 g) were  
24 enclosed in square bags (140 mm wide × 170 mm deep) of thin  
25 plastic made of nylon 15 μm/linear low-density polyethylene 60  
26 μm.

27  
28 *Lipid oxidation in mayonnaise and taste sensory evaluation.*  
29 Mayonnaise formulations were autoxidized at 55°C for 7 days in



1 the dark to measure the peroxide value (PV). After oxidization,  
2 lipids in the mayonnaise were extracted using the Bligh and Dyer  
3 method.<sup>22)</sup> PV was determined according to the Japan Oil  
4 Chemists Society (JOCS) official method 2.5.2.2-2013.

5 In addition to the autoxidation, mayonnaise formulations were  
6 also photo-oxidized at 25°C for 7 days under a fluorescent lamp  
7 at 500–600 lx for sensory testing. Taste, as evaluated by  
8 eight-trained panelists, was scored from 0 to 7, with higher  
9 numbers indicating better taste, on the basis of a score of 7 as  
10 the standard taste of mayonnaise stored at 4°C for 7 days in the  
11 dark.

12  
13 *DPPH radical-scavenging activity.* Egg white protein  
14 was dispersed in 1% (w/v) taurocholate and then sonicated with a  
15 probe-type homogenizer (Ultra S, VP-5S; Taitec, Saitama). Egg  
16 white protein hydrolysate (EP-1) and the amino acid mixture  
17 were dissolved in 6N HCl/DMSO (0.016:1, v/v) and 6N  
18 HCl/DMSO (0.056:1, v/v), respectively. The three resulting  
19 solutions were visually clear. The concentration of these samples  
20 was 20 mg/mL, and the negative controls and dilutions were  
21 prepared using the corresponding vehicles.

22 The scavenging activity of these samples on DPPH radicals  
23 was evaluated using a modification of a method described  
24 previously.<sup>23)</sup> The solutions (20 µL) of egg white protein, egg  
25 white protein hydrolysate, and the amino acid mixture were  
26 incubated with DPPH working solution consisting of 90 µL of  
27 100 mM DPPH-ethanol and 90 µL of 100 mM acetate buffer (pH  
28 4.0) in each well of a 96-well plate for 30 min at room  
29 temperature. The supernatants (100 µL) were centrifuged at

1 1,600 g for 10 min at 4°C, transferred to a new 96-well plate, and  
2 then diluted 2-fold with distilled water. The DPPH content was  
3 analyzed at 510 nm using a microplate reader (Tecan Group,  
4 Männedorf, Switzerland). Trolox-ethanol was used as a positive  
5 control.

6  
7 *Fe<sup>2+</sup>-chelating activity.* Fe<sup>2+</sup>-chelating activity was  
8 evaluated using a modification of a previously described  
9 method.<sup>24,25)</sup> The solutions (75 µL) of egg white protein, egg  
10 white protein hydrolysate, the amino acid mixture, and negative  
11 controls and dilutions as described previously were mixed with  
12 75 µL of ammonium iron (II) sulfate hexahydrate at 100 µM  
13 and 600 µL of 100 mM acetate buffer (pH 4.0). After incubation  
14 at 55°C for 30 min, 7.5 µL of 100% TCA were added to 150 µL of  
15 the reaction mixture and then centrifuged at 9,600 g for 10 min at  
16 4°C. The supernatants (100 µL) were incubated with 80 µL of  
17 10% (w/v) ammonium acetate and 20 µL of the ferrous iron color  
18 indicator consisting of 6.1 mM ferrozine and 14.4 mM  
19 neocuproine, which could be dissolved by the addition of several  
20 drops of 6N HCl, in 96-well plates for 5 min at room temperature,  
21 and then analyzed at 560 nm using the microplate reader.  
22 EDTA-2Na was used as a positive control. Vehicle alone was  
23 used as the negative control for each sample.

24 During the incubation, there was a concern regarding  
25 reductions of Fe<sup>2+</sup> content by autoxidation. Concomitant with the  
26 measurement of chelating activity, the oxidative stability of Fe<sup>2+</sup>  
27 in each negative control solution was evaluated. In addition, due  
28 to the possible conversion of Fe<sup>2+</sup> to Fe<sup>3+</sup> caused by the egg  
29 white components, supernatants (100 µL) of samples were also

1 mixed with 100  $\mu$ L of 5-sulfosalicylic acid dihydrate at 200  $\mu$ M  
2 in 96-well plates for 10 min at room temperature, followed by  
3 analysis at 510 nm using a microplate reader via a modification  
4 of a previously described method for evaluating  $\text{Fe}^{3+}$ .<sup>26)</sup> Because  
5  $\text{Fe}^{3+}$  did not cause a color change in the reaction using the  
6 ferrous iron color indicator, the production of  $\text{Fe}^{3+}$  induced by  
7 the components might create the mistaken impression of  
8 effective  $\text{Fe}^{2+}$ -chelating activity.

9  
10 *Statistical analysis.* The data were analyzed using  
11 one-way ANOVA with Dunnett test or with Tukey–Kramer test.  
12 P-values < 0.05 were considered significant.

## 14 **Results**

### 15 *Lipid oxidation in the acidic egg yolk solution*

16 We measured the fluorescence of substances produced by the  
17 oxidation of acidic egg yolk solution as a mayonnaise model. The  
18 fluorescent substances are thought to be products of aldehyde  
19 groups in volatile compounds and amino groups as described  
20 below.

21 Fig. 1A shows a GC chromatogram of the volatile compounds  
22 formed by oxidation in the acidic egg yolk solution at 55°C for  
23 72 h. The three main peaks were assigned by comparing MS  
24 spectra with those of the database library as follows: peak a,  
25 hexanal (Fig. 1B); peak b, 2-pentylfuran (Fig. 1B); and peak c,  
26 acetic acid (data not shown).

27 We plotted the fluorescence intensity of substances against GC  
28 responses of the first two compounds produced by the oxidation  
29 of acidic egg yolk solution at 55°C for 72 h under variable

1 conditions (pH 4.0 or 7.0 with EDTA at 0 or 25  $\mu$ M). Positive  
2 correlations were found between the fluorescence intensity and  
3 the formation of hexanal ( $R^2 = 0.9757$ ) or 2-pentylfuran ( $R^2 =$   
4  $0.7318$ ) (Fig. 1C). In particular, the fluorescence intensity  
5 exhibited a strong correlational relationship with the hexanal  
6 formation.

7

8 *The inhibitory effects of egg white protein/hydrolysate/amino*  
9 *acid mixture on lipid oxidation in acidic egg yolk solution as a*  
10 *mayonnaise model*

11 We evaluated the antioxidative effect of egg white components  
12 using acidic egg yolk solution as a mayonnaise model.

13 The fluorescence intensity in acidic egg yolk solution was  
14 increased during incubation in a time-dependent manner (Fig. 2A,  
15 Control). EDTA (0.0047%), positive control, significantly  
16 decreased the intensity at 88 h (one-way ANOVA,  $p < 0.05$ ) (Fig.  
17 2A). Egg white protein, the hydrolysate, and the amino acid  
18 mixture also trended to decrease the fluorescence intensity in a  
19 concentration-dependent manner over the range of  
20 0.0125%–0.1% (Figs 2B-E).

21 As shown in Figs 2 B-E, egg white hydrolysate and the amino  
22 acid mixture more strongly decreased the fluorescence intensity  
23 than egg white protein (The values at 88 h,  $p < 0.05$ ). Among the  
24 three egg white components, the hydrolysate at 0.1% most  
25 effectively decreased the intensity (The values at 88 h,  $p < 0.05$ )  
26 (Fig. 2E).

27

28 *DPPH radical-scavenging activity of egg white*  
29 *protein/hydrolysate/amino acid mixture*

1 As described above, egg white components showed the  
2 antioxidative effect in acidic egg yolk solution as a mayonnaise  
3 model. Because the lipid oxidation is further accelerated by  
4 radicals produced from lipid peroxide,<sup>27)</sup> radical-scavenging  
5 activity is thought as a possible mechanism for the antioxidative  
6 effect. DPPH was appropriately used for evaluating the activity  
7 as described below. EDTA showed no effect at all on the DPPH  
8 radical-scavenging activity (data not shown). Trolox is  
9 commonly used as a positive control. As shown in Fig. 3A, the  
10 remaining levels of DPPH radicals (%) (Y) were plotted against  
11 the Trolox concentrations (mM) (X), revealing a negative  
12 correlation under the experimental condition used ( $R^2 = 0.9907$ ).

13 Egg white protein, the hydrolysate, and the amino acid mixture  
14 reduced DPPH radical levels in a concentration-dependent  
15 manner. The amino acid mixture had the strongest effect on  
16 DPPH radical among the three components at 2.0% (Fig. 3B).  
17 When the DPPH radical-scavenging activity of these components  
18 at 2.0% was evaluated based on that of Trolox using the formula  
19  $Y = 166.66X$ , the values were  $0.155 \pm 0.011$  mM for protein,  
20  $0.126 \pm 0.004$  mM for the hydrolysate, and  $0.293 \pm 0.026$  mM for  
21 the amino acid mixture.

22

23 *Fe<sup>2+</sup>-chelating activity of egg white protein/hydrolysate/amino*  
24 *acid mixture*

25 As iron release caused lipid oxidation, the iron chelating  
26 effect would be one of the possible mechanisms of the  
27 antioxidative effect. An extremely low concentration (50  $\mu$ M,  
28 0.0186 mg/mL) of EDTA-2Na as a positive control chelated  
29 nearly all of the Fe<sup>2+</sup> content (Fig. 4). Among the three egg white

1 components, the hydrolysate exhibited the greatest  
2  $\text{Fe}^{2+}$ -chelating activity at 1.0% (Fig. 4), with approximately 50%  
3 of  $\text{Fe}^{2+}$  chelated after incubation for 30 min. Egg white protein  
4 and the amino acid mixture had little to no  $\text{Fe}^{2+}$ -chelating  
5 activity.

6 No oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  was observed during incubation  
7 for 30 min (data not shown).

8  
9 *The inhibitory effect of the hydrolysate on lipid oxidation in*  
10 *mayonnaise*

11 We evaluated the antioxidative effect of egg white components  
12 on real mayonnaise. After incubation for 7 days in the dark at  
13  $4^{\circ}\text{C}$ , the PV in mayonnaise was approximately zero (data not  
14 shown), whereas the values at  $55^{\circ}\text{C}$  reached approximately 6 (Fig.  
15 5, Control). EDTA significantly decreased the PV. The  
16 hydrolysate (EWH) also significantly decreased the value over  
17 the range of 0.09%–0.9% (Fig. 5).

18 Fig. 6 shows the results of the sensory taste evaluation of  
19 mayonnaise after incubation for 7 days. Under the condition of  
20  $25^{\circ}\text{C}$  with light at 500–600 lx (Control), the average value of  
21 taste score was low (2.5). EDTA significantly inhibited the  
22 deterioration of mayonnaise, with the average score increasing to  
23 5.5. The hydrolysate at 0.09% and 0.45% also significantly  
24 inhibited the deterioration (average scores, 4.25 and 5.0,  
25 respectively), but no effect was observed at a higher  
26 concentration (0.9%).

27 During the evaluating period, time-dependent viscosity  
28 changes were not significantly different among the mayonnaise  
29 samples (data not shown). Visible oil layer separation was also

1 not observed in any samples (data not shown).

2

3 *The effect of various acids on lipid oxidation in acidic egg*  
4 *yolk solution*

5 In general, mayonnaise is adjusted to an acidic condition using  
6 vinegar containing acetic acid. However, as described above,  
7 acetic acid itself may accelerate the lipid oxidation. We  
8 evaluated the effect of various acids (six organic acids and two  
9 inorganic acids: hydrochloric acid and phosphoric acid) on lipid  
10 oxidation in the egg yolk solution and then compared them with  
11 acetic acid. Each egg yolk solution was adjusted to pH 4.0 with  
12 eight corresponding acids. Among the organic acids tested, citric  
13 acid and tartaric acid significantly reduced the fluorescence  
14 intensity after incubation for both 40 h and 88 h compared with  
15 the effect of acetic acid (Fig. 7).

16 Fig. 8 presents the  $\text{Fe}^{2+}$ -chelating activity of citric acid at pH  
17 4.0. Citric acid significantly reduced the amounts of  $\text{Fe}^{2+}$ . At  
18 1000  $\mu\text{M}$ , the amount of  $\text{Fe}^{2+}$  was reduced to approximately 60%  
19 of the initial level. Citric acid at the same concentrations showed  
20 no effect at all on the DPPH radical scavenging activity (data not  
21 shown).

22

## 23 **Discussion**

24

25 In the present study, we evaluated the effect of egg  
26 white-derived components such as egg white protein, the  
27 hydrolysate, and the amino acid mixture on lipid oxidation in  
28 mayonnaise.

29 We constructed a mayonnaise model using acidic egg yolk

1 solution (pH 4.0) and then assessed the antioxidant action of egg  
2 white-derived components. The formation of fluorescent lipid  
3 peroxidation products was measured as an index of lipid  
4 oxidation in the acidic egg yolk solution. The fluorescent  
5 products are known to be produced from a Schiff base of an  
6 amino group and an aldehyde group.<sup>20)</sup> Some volatile compounds  
7 were produced during the oxidation of acidic egg yolk solution.  
8 The amounts of hexanal (caproaldehyde) and 2-pentylfuran  
9 compounds among the volatile compounds were correlated with  
10 an increase in fluorescence intensity (Fig. 1C). Hexanal was  
11 reported to form fluorescent products with lysine.<sup>28)</sup> Another  
12 volatile compound, 2-pentylfuran, was reported as one of the  
13 oxidative degradation products produced from linoleic acid,<sup>29)</sup> a  
14 major polyunsaturated fatty acid in egg yolk, and as a potential  
15 marker of lipid peroxidation. However, it does not have an  
16 aldehyde group within its molecule. The production of  
17 2-pentylfuran would be in appearance correlated with an increase  
18 in fluorescent products. Thus, in the present study, hexanal  
19 production would involve the production of fluorescent products.

20 Some proteins and amino acids are known as natural  
21 antioxidative components with iron-chelating and/or  
22 radical-scavenging activity. Amino acids<sup>30)</sup> such as histidine,  
23 lysine, and cysteine and proteins such as egg albumin,<sup>31)</sup> soy  
24 protein,<sup>10)</sup> casein,<sup>32)</sup> and gelatin<sup>33)</sup> inhibited the oxidation of  
25 linoleic acid in various model systems. In addition, the  
26 hydrolysate/peptides of some proteins were more effective in  
27 inhibiting lipid oxidation than their parental proteins. After the  
28 milk was treated with trypsin, the oxidation of milk fat was  
29 inhibited compared with before the treatment, suggesting that



1 casein hydrolysate exerted stronger antioxidative effects.<sup>34)</sup> Soy  
2 protein hydrolysate more effectively inhibited the oxidation of  
3 linoleic acid than soy protein.<sup>35)</sup> Egg yolk protein hydrolysate  
4 displayed stronger antioxidative effects on the oxidation of  
5 linoleic acid in cookies than egg yolk protein and amino acids.<sup>11)</sup>  
6 In agreement with these studies, we found that egg white protein  
7 hydrolysate most strongly inhibited lipid oxidation (Fig. 2E).  
8 The antioxidative effect of the hydrolysate was reported to be  
9 dependent on the variety of enzymes used to cleave peptide  
10 bonds, thereby indicating that the antioxidative effects of  
11 hydrolysate/peptides would be affected by the amino acid  
12 residues and the terminal sites.<sup>36)</sup>

13 To investigate the mechanism underlying the inhibitory effect  
14 of egg white hydrolysate on the oxidation of acidic egg yolk  
15 solution, we evaluated the following two points:  
16 radical-scavenging and Fe<sup>2+</sup>-chelating activity. In the present  
17 study, among the three egg white components, amino acid  
18 mixture displayed the strongest DPPH radical-scavenging  
19 activity (Fig. 3B). Amino acids such as histidine,<sup>37,38)</sup> and  
20 cysteine<sup>39)</sup> were previously reported to have DPPH  
21 radical-scavenging activity. The amino acid mixture used in the  
22 present study contained these amino acids, and thus they also  
23 would exert the strongest effect under acidic conditions. As  
24 described previously, some studies<sup>11,34,35,40)</sup> reported that  
25 hydrolysate exhibited stronger inhibitory effects than the  
26 parental proteins on lipid oxidation and that the DPPH  
27 radical-scavenging activity of the hydrolysate was one of the  
28 mechanisms responsible for the stronger antioxidative effect.  
29 Egg white hydrolysate also displayed DPPH radical-scavenging

1 activity. However, the effect tended to be weaker than that of  
2 protein, although there was no statistically difference between  
3 the two. The results suggested that the main inhibitory effect of  
4 the hydrolysate on lipid oxidation in the acidic egg yolk solution  
5 was not caused by radical-scavenging activity.

6 We evaluated  $\text{Fe}^{2+}$ -chelating activity as another possible  
7 mechanism for the antioxidative action of egg white hydrolysate.  
8 The  $\text{Fe}^{2+}$ -chelating activity tended to be in the order of  
9 hydrolysate  $\gg$  amino acid mixtures = protein, suggesting that  
10 the  $\text{Fe}^{2+}$ -chelating activity explained the antioxidative effect of  
11 the hydrolysate (Fig. 4). Thus, the results suggested that the  
12 inhibitory effect of the hydrolysate on lipid oxidation in the  
13 acidic egg yolk solution could be mainly due to its  
14  $\text{Fe}^{2+}$ -chelating activity. During the evaluation of  $\text{Fe}^{2+}$ -chelating  
15 activity,  $\text{Fe}^{2+}$  was extremely stable under an acidic condition in  
16 the current study, in agreement with previous reports.<sup>41)</sup> Thus,  
17 the autoxidation of  $\text{Fe}^{2+}$  did not interfere with the measurement  
18 of  $\text{Fe}^{2+}$ -chelating activity.

19 The enzymatic hydrolysate of egg white albumin was  
20 previously reported to inhibit the oxidation of linoleic acid in  
21 ethanol/phosphate buffer<sup>42)</sup> and corn oil emulsion.<sup>25)</sup> The  
22 hydrolysate displayed strong  $\text{Fe}^{2+}$ -chelating activity, and this  
23 activity was believed to explain the antioxidative effect,  
24 agreeing with our results. Some peptides in the hydrolysate have  
25 been considered to have antioxidative effects. Ala-His-Lys was  
26 previously identified as a candidate substance responsible for  
27 the antioxidative action of protein hydrolysate.<sup>42)</sup> In the present  
28 study, the antioxidative and chelating activities of peptides in  
29 egg white hydrolysate were not clarified. Thus, a detailed

1 determination of the amino acid residue involved in these effects  
2 requires further study.

3 We confirmed the inhibitory effect of egg white hydrolysate on  
4 lipid oxidation in real mayonnaise by determining the PV. The  
5 hydrolysate significantly inhibited lipid oxidation in a  
6 concentration-dependent manner. Hydrolysate at 0.45 and 0.9%  
7 exerted a similar effect as 0.01% EDTA, suggesting that the  
8 hydrolysate have the potential to inhibit lipid oxidation as an  
9 alternative of EDTA (Fig. 5). In addition to their effect on  
10 oxidative stability, the rating in the sensory evaluation of  
11 mayonnaise was also highest for the hydrolysate at 0.45% as well  
12 as EDTA at 0.01% (Fig. 6). Although the rating for hydrolysate at  
13 0.9% was lower than that at 0.45%, off-flavors such as oxidized  
14 flavors were not observed in mayonnaise.

15 In mayonnaise containing egg white hydrolysate at 0.9%, the  
16 panelists sensed a bitter taste, which was significantly different  
17 from the standard taste. In fact, the bitter taste of egg white  
18 hydrolysate has been previously reported.<sup>43)</sup> These results  
19 suggest that at the higher concentration, the taste of the  
20 hydrolysate themselves was responsible for the lower rating in  
21 the sensory evaluation. On the basis of these results, a  
22 hydrolysate concentration of 0.45% would be necessary to obtain  
23 the same inhibitory effect as 0.01% EDTA. The concentration of  
24 the hydrolysate did not affect the flavor, taste, color tone, or  
25 physical properties of mayonnaise. Thus, the egg white  
26 hydrolysate would be useful components as natural antioxidants  
27 in inhibiting deterioration of mayonnaise

28 Although acetic acid is generally used to make mayonnaise, it  
29 has pro-oxidative activity.<sup>12)</sup> The use of other organic acids may

1 further enhance resistance to lipid oxidation in mayonnaise, as  
2 organic acids such as citric acid have been reported to have  
3 antioxidative and/or iron chelating activity.<sup>13,14)</sup> In the present  
4 study, we evaluated the antioxidant action of eight acids, namely  
5 six organic acids, hydrochloric acid, and phosphoric acid.  
6 Among the acids tested, citric acid effectively inhibited lipid  
7 oxidation in the acidic egg yolk solution (Fig. 7). Citric acid  
8 also displayed Fe<sup>2+</sup>-chelating activity under the acidic condition,  
9 although the effect was weaker than that of EDTA (Fig. 8). These  
10 results suggested that the effect of citric acid on lipid oxidation  
11 in the acidic egg yolk solution was caused by the chelating  
12 activity. From the point of view of microorganism propagation,  
13 in the processing of mayonnaise, the whole acetic acid cannot be  
14 replaced to citric acid because the antimicrobial action of citric  
15 acid was lower than that of acetic acid.<sup>44)</sup> However, acetic acid  
16 may be partly replaced by citric acid as follows. It may be  
17 possible to use the lemon fruit juice, including the citric acid,  
18 for the production of mayonnaise. The oxidative stability of  
19 lipids in real mayonnaise using citric acid together with acetic  
20 acid deserves further study.

21 In conclusion, to estimate the inhibitory effect of egg white  
22 components on lipid oxidation in mayonnaise, we used acidic egg  
23 yolk solution as a simple model system. Oxidation was measured  
24 using fluorescence intensity, which was correlated with  
25 increasing hexanal formation during the incubation. Among the  
26 egg white components tested, hydrolysate displayed the strongest  
27 inhibiting effect. The antioxidant activity of the egg white  
28 hydrolysate could be mainly due to its Fe<sup>2+</sup>-chelating activity.  
29 The hydrolysate also inhibited lipid oxidation based on

1 measurements of the PV in real mayonnaise and significantly  
2 suppressed the appearance of off-flavor in sensory taste  
3 experiments. The present study indicates that the egg white  
4 hydrolysate is a natural product with the potential to effectively  
5 inhibit the degradation of mayonnaise.

6

#### 7 **Authors contributions**

8

9 H.K. designed the study. H.K. and E.K.-N. wrote the  
10 manuscript. R.S. and S.Y. performed lipid oxidation in  
11 mayonnaise and taste sensory evaluation and analyzed the data.  
12 E.K.-N. performed the experiments of DPPH and iron chelate and  
13 analyzed the data. All authors contributed to the critical revision  
14 of the manuscript.

15

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17

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23 ([www.enago.jp](http://www.enago.jp)) for the English language review.

24

#### 25 **Disclosure statement**

26

27 The authors declare no conflicts of interest.

28

29

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For Peer Review

1 **Figure legends**

2

3 **Fig. 1 Volatile compounds and fluorescent products in the**  
4 **oxidation of acidic egg yolk solution as a model system of**  
5 **mayonnaise.**

6 (A) GC profile in acidic egg yolk solution after incubation  
7 at 55°C for 72 h. Peak a, hexanal; peak b, 2-pentylfuran; peak c,  
8 acetic acid. (B) Mass spectra of peaks a and b. (C) Relationship  
9 between fluorescence intensity at Ex. 360 nm/Em. 440 nm in egg  
10 yolk solution and GC-MS responses of volatile compounds  
11 (hexanal and 2-pentylfuran) after incubation at 55°C for 72 h  
12 under variable pH (4.0 or 7.0) and ethylenediaminetetraacetic  
13 acid concentrations (0 or 25  $\mu$ M). The data are presented as the  
14 mean  $\pm$  SD (n = 3).

15

16 **Fig. 2 Inhibitory effects of three egg white components on**  
17 **lipid oxidation in acidic egg yolk solution.**

18 Acidic egg yolk solutions were incubated at 55°C for the  
19 indicated times, and the fluorescence intensity at Ex. 360 nm/Em.  
20 440 nm was measured. (A) Negative control (no component),  
21 open circles; positive control (ethylenediaminetetraacetic acid at  
22 0.0047%), open triangles. The data are presented as the mean  $\pm$   
23 SD (n = 3). The asterisk for the values at 88 h indicates  
24 significant differences (one-way ANOVA,  $p < 0.05$ ). (B–E) Egg  
25 white protein, filled circles; hydrolysate, filled squares; amino  
26 acid mixture, filled triangles. These components were added to  
27 acidic egg yolk solution at concentrations of 0.0125% (B),  
28 0.025% (C), 0.05% (D), and 0.1% (E). The data are presented as  
29 the mean  $\pm$  SD (n = 3). The asterisks for the values at 88 h

1 indicate significant differences (one-way ANOVA with  
2 Tukey–Kramer test,  $p < 0.05$ ).

3

4 **Fig. 3 DPPH radical-scavenging activity under acidic**  
5 **conditions.**

6 DPPH radical scavenging activity. DPPH/ethanol was  
7 mixed with trolox as a positive control or egg white  
8 components/acetate buffer (pH 4.0) and then incubated at room  
9 temperature for 30 min. DPPH was measured at 510 nm. (A)  
10 Trolox. (B) Egg white protein, filled circles; hydrolysate, filled  
11 squares; amino acid mixture, filled triangles. The remaining  
12 DPPH radical content was expressed as the percentage of the  
13 value of the control. The data are presented as the mean  $\pm$  SD of  
14 eight wells. The asterisks for the values at a concentration of  
15 2.0% indicate significant differences (one-way ANOVA with  
16 Tukey–Kramer test,  $p < 0.05$ ).

17

18 **Fig. 4 Fe<sup>2+</sup>-chelating activity of three egg white components**  
19 **under acidic conditions.**

20 Fe<sup>2+</sup>-chelating activity. Fe<sup>2+</sup> was mixed with egg white  
21 components/acetate buffer (pH 4.0) and then incubated at 55°C  
22 for 30 min, after which the ferrous iron content was measured  
23 via ferrozine colorimetry at 560 nm. Ethylenediaminetetraacetic  
24 acid (EDTA) at 25  $\mu$ M (0.0009%) and 50  $\mu$ M (0.0019%) as a  
25 positive control, open circle. Egg white protein, filled circles;  
26 hydrolysate, filled squares; amino acid mixture, filled triangles.  
27 The remaining Fe<sup>2+</sup> amounts were expressed as a percentage of  
28 the control. The data are present as the mean  $\pm$  SD of four wells.  
29 The asterisks for the values at a concentration of 1.0% indicate

1 significant differences (one-way ANOVA with Tukey–Kramer  
2 test,  $p < 0.05$ ).

3

4 **Fig. 5 Inhibitory effect of egg white hydrolysate on lipid**  
5 **oxidation in mayonnaise.**

6 Mayonnaise was prepared according to the formula shown  
7 in Table 2. It was incubated at 55°C for 7 days in the dark. The  
8 effect of egg white hydrolysate (EWH) at the indicated  
9 concentrations was evaluated by measuring the peroxide value  
10 (PV). Ethylenediaminetetraacetic acid (EDTA) at 0.01% was used  
11 as a positive control. The data are presented as the mean  $\pm$  SD ( $n$   
12 = 3). The asterisks indicate significant differences from control  
13 (one-way ANOVA with Dunnett test,  $p < 0.05$ ).

14

15 **Fig. 6 Inhibitory effect of egg white hydrolysate on**  
16 **deterioration of mayonnaise by lipid oxidation.**

17 Mayonnaise was incubated at 25°C for 7 days under light.  
18 The effect of egg white hydrolysate (EWH) at the indicated  
19 concentrations was evaluated by eight-trained sensory taste  
20 panels. EDTA at 0.01% was used as a positive control. Each  
21 average score was expressed at a lower side. The asterisks  
22 indicate significant differences from control (one-way ANOVA  
23 with Dunnett test,  $p < 0.05$ ).

24

25 **Fig. 7 Inhibitory effects of various acids on lipid oxidation**  
26 **in the acidic egg yolk solution.**

27 The pH of egg yolk solutions was adjusted to 4.0 with eight  
28 acids, and followed by incubation at 55°C for 40 h or 88 h. The  
29 fluorescence intensity at Ex. 360 nm/Em. 440 nm was measured.

1 The data are presented as the mean  $\pm$  SD ( $n = 3$ ). The asterisks  
2 indicate significant differences from the values of acetic acid  
3 (one-way ANOVA with Dunnett test,  $p < 0.05$ ).

4

5 **Fig. 8 Fe<sup>2+</sup>-chelating activity of citric acid under acidic**  
6 **conditions.**

7 Citric acid was used at a concentration of 250–1000  $\mu$ M  
8 (0.005%–0.02%). Fe<sup>2+</sup> was mixed with citric acid/acetate buffer  
9 (pH 4.0) and then incubated at 55°C for 30 min, and the ferrous  
10 iron contents were measured via ferrozine colorimetry at 560 nm.  
11 The data are presented as the mean  $\pm$  SD of four wells. The  
12 asterisks indicate significant differences from control (one-way  
13 ANOVA with Dunnett test,  $p < 0.05$ ).

14

15 **Graphical abstracts**

16 Proposed mechanism of lipid oxidation in mayonnaise and  
17 antioxidative activity of egg white protein hydrolysate.

18 “-P<”, phosphate group.

**Table 1** Amino acid composition of egg white protein and hydrolysate

Amino acid	Protein	Hydrolysate (EP-1) <sup>*3</sup>
Threonine	44	46
Tyrosine	37	39
Phenylalanine	59	60
Cysteine	27	26
Methionine	39	39
Valine	68	70
Isoleucine	52	53
Leucine	82	84
Lysine	72	73
Tryptophan	16	15
Histidine	23	27
Aspartic acid <sup>*1</sup>	99	92
Serine	64	68
Glutamic acid <sup>*2</sup>	121	123
Proline	34	29
Glycine	34	33
Alanine	70	66
Arginine	59	57
Total	1000	1000
(mg/g protein)		

<sup>\*1</sup> Total of asparagine and aspartic acid.

<sup>\*2</sup> Total of glutamine and glutamic acid.

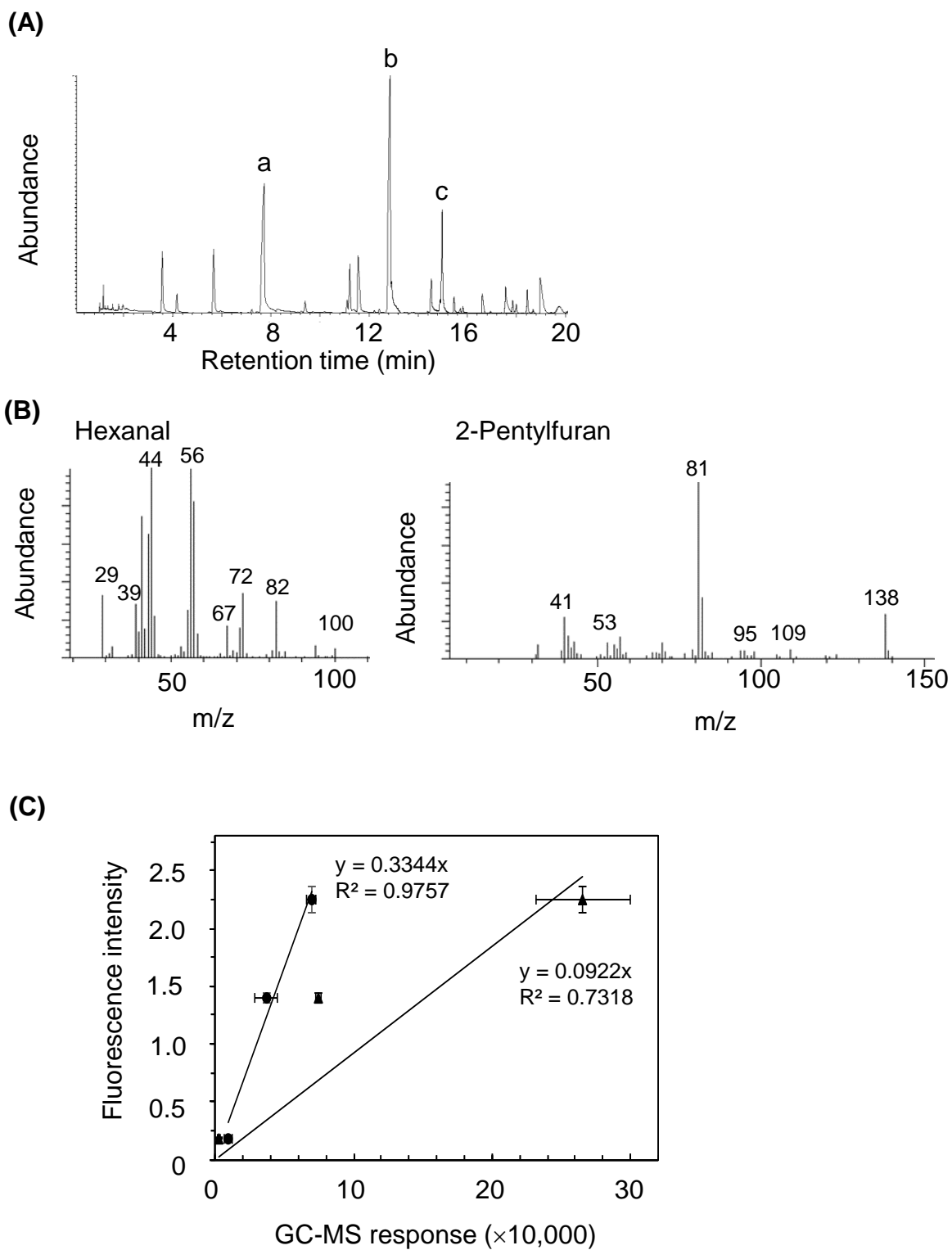
<sup>\*3</sup> The hydrolysate is a commercial product. The amino acid mixture was compounded in the same formulation ratio.



**Table 2** Formulation of mayonnaise

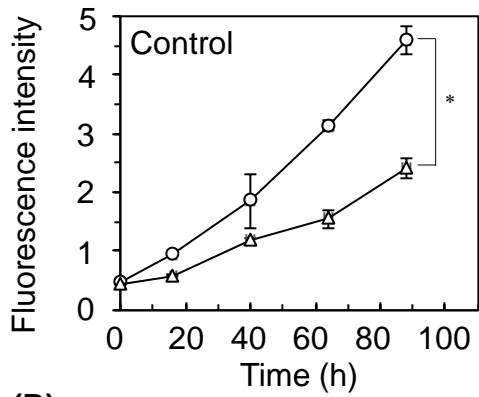
	Control	EDTA · Ca · 2Na	Egg white hydrolysate		
			0.09%	0.45%	0.9%
Vegetable oil <sup>*1</sup>	7.5	7.5	7.5	7.5	7.5
10% salted egg yolk	1.0	1.0	1.0	1.0	1.0
Vinegar <sup>*2</sup>	0.7	0.7	0.7	0.7	0.7
Water	0.720	0.719	0.711	0.675	0.630
Salt	0.05	0.05	0.05	0.05	0.05
Sodium glutamate	0.03	0.03	0.03	0.03	0.03
Mustard	0.006	0.006	0.006	0.006	0.006
Egg-white hydrolysate	-	-	0.009	0.045	0.090
EDTA · Ca · 2Na	-	0.001	-	-	-
<b>Total (kg)</b>	<b>10.006</b>	<b>10.006</b>	<b>10.006</b>	<b>10.006</b>	<b>10.006</b>

<sup>\*1</sup>Canola oil/soybean oil (1:1). <sup>\*2</sup>Acetic acid 9%.

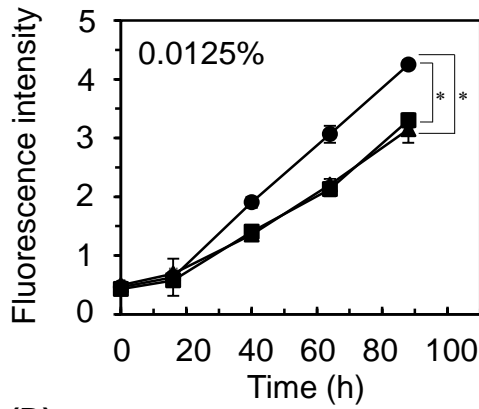


↑Figure 1  
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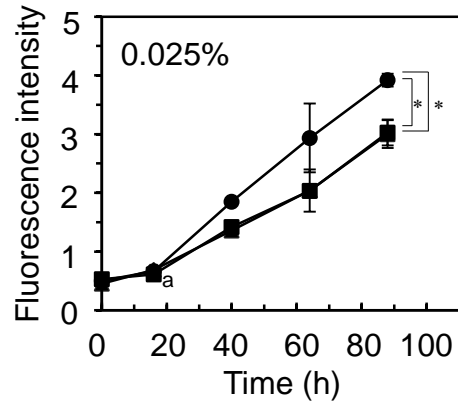
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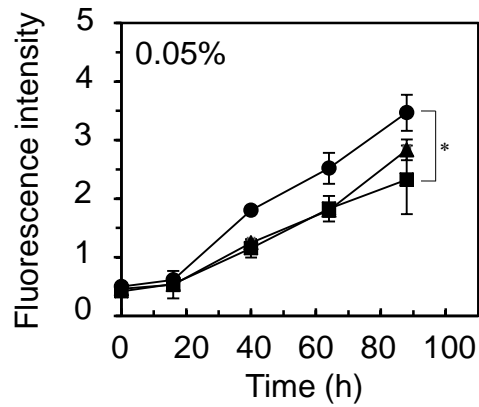
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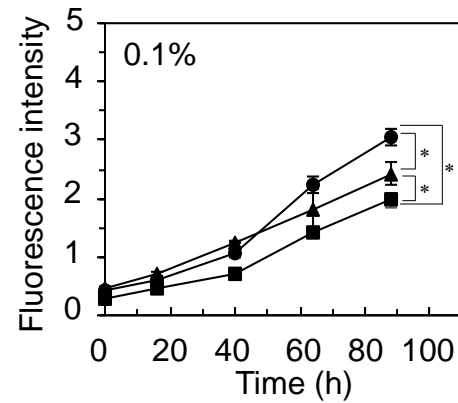
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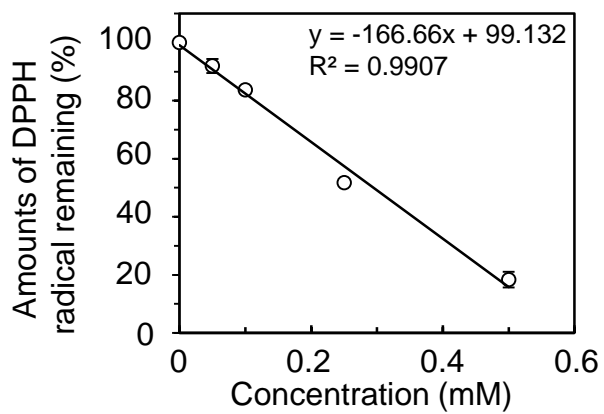


(E)

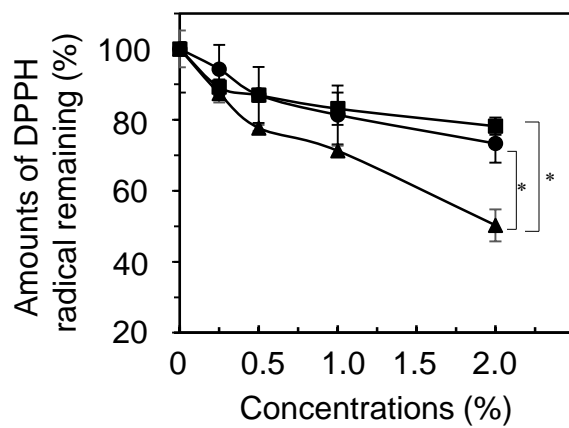


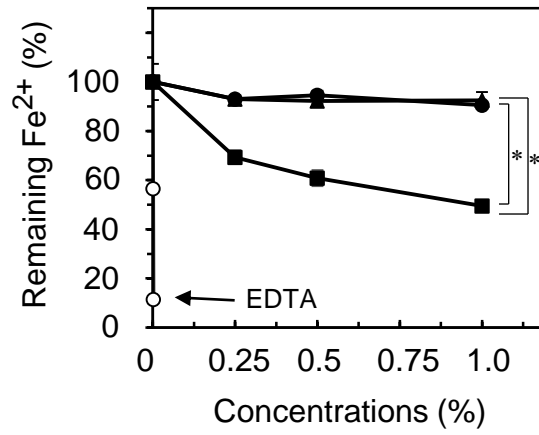
↑Figure 2  
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(A)

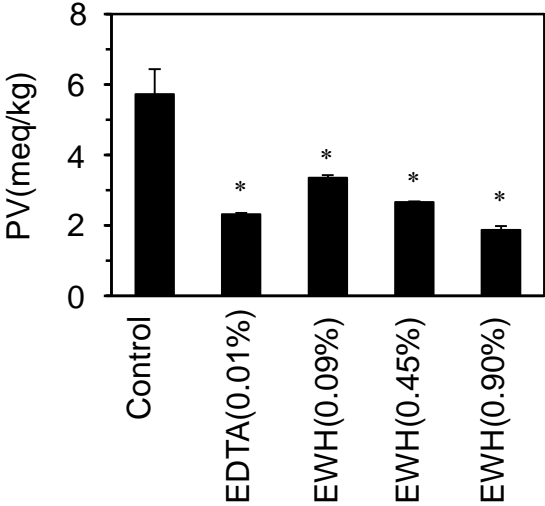


(B)

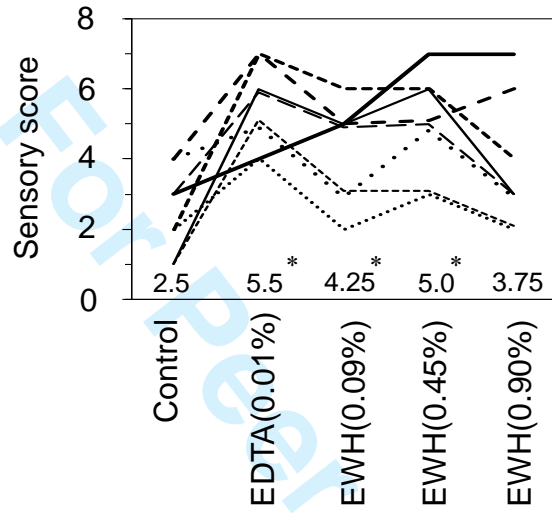




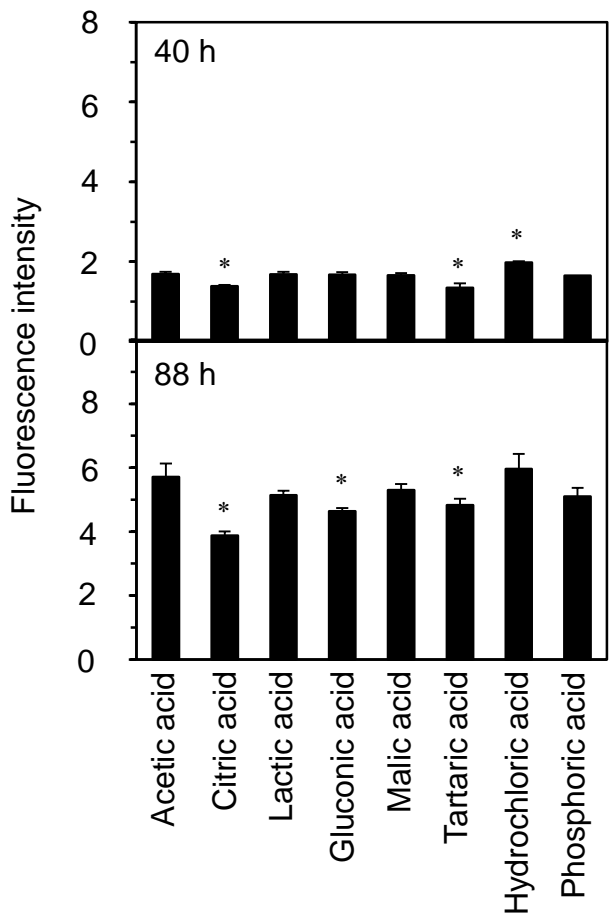
↑Figure 4  
H. Kobayashi et al.



↑Figure 5  
H. Kobayashi et al.

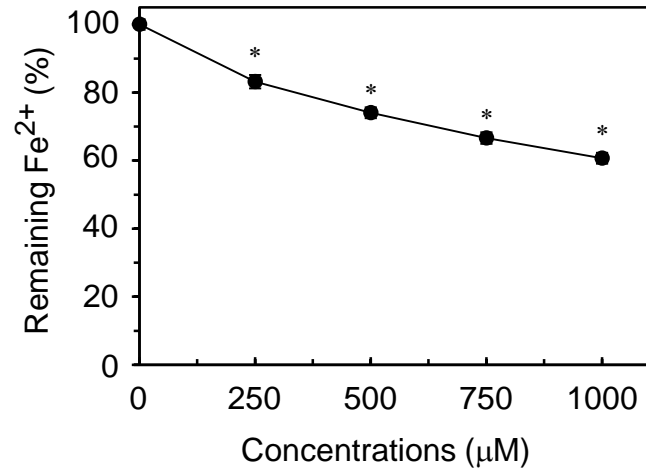


↑Figure 6  
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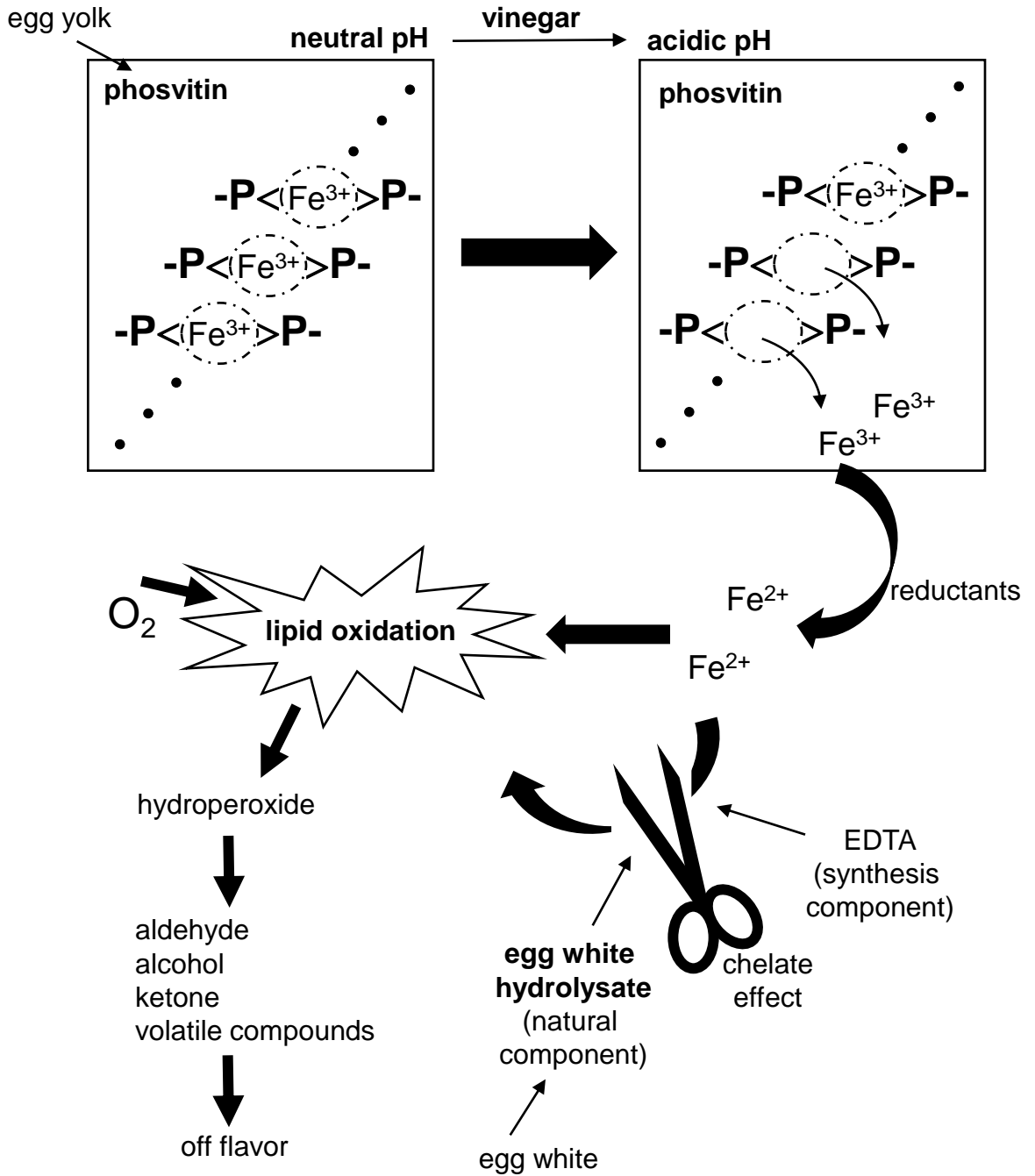


↑Figure 7  
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↑Figure 8  
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**Graphical abstract**  
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