Random-centroid Optimization Reveals the Strongest Superoxide Anion Radical Scavenging Activity of Maltose- and Ribose-conjugated Chicken Myofibrillar Protein

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Random-centroid optimization (RCO) was applied to determine the optimum preparative conditions for glycated chicken myofibrillar proteins (Mfs) conjugated to maltose or ribose. The optimization targets were antioxidative ability against superoxide anion radicals (O₂⁻) and a minimum 60% solubility in low ionic strength medium. Four optimization parameters, temperature, relative humidity (RH), reaction time, and quantity of maltose or ribose, were selected and 13 experimental conditions were obtained (using the RCO program). The examinations were independently assessed according to individual vertex values. The obtained optimal conditions for maltose and ribose were determined to be 61 and 38°C, 38 and 39% RH, 33.9 and 3.80 h reaction time, and a sugar to protein mixing ratio (w/w) of 5.59 and 10.4 (w/w), respectively, and O₂⁻ scavenging activities of each conjugate reached the maximum of 274 ± 86 (n=3) and 368 ± 120 (n=3) units of superoxide dismutase/g of protein.

Keywords: myofibrillar proteins, maltose, ribose, maillard reaction, glycation, antioxidant, random-centroid optimization

Introduction

Functional alterations to the Maillard reaction can result from glycation of chicken myofibrillar proteins (Mfs) with sugars (Nishimura et al., 2011a,b; Isono et al., 2012; Nishimura et al., 2015; Nishimura and Saeki, 2016). Glycation with maltose confers improved solubility in a low ionic strength medium, higher thermal stability (Nishimura et al., 2011a,b) and antioxidant abilities (Nishimura et al., 2011b), as measured by superoxide anion radical (O₂⁻) scavenging activities (SOSA). Antioxidant ability is attributed to the primary structure—the specific peptide sequence of the glycated chicken Mfs—irrespective of the protein shape (Isono et al., 2012). In the previous study (Nishimura and Saeki, 2016), the optimum preparative conditions (57°C, 37% relative humidity (RH), 37.2 h reaction time, and a maltose mixing ratio of 5.43 (w/w)) to obtain glycated chicken Mfs were determined. The conditions were designed to produce the strongest antioxidant ability against hydroxyl radicals (•OH) using random-centroid optimization (RCO) (Nishimura et al., 1997, 1998; Goto et al., 2000; Nishimura et al., 2001; Goto et al., 2004; Nakai et al., 2009; Yan and Wang, 2015; Nishimura and Saeki, 2016). Optimized glycated chicken Mfs retained thermal gel forming ability, and the gel retained approximately one-half of its antioxidant activity after heating (Nishimura and Saeki, 2016).

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ESR, electron spin resonance; IC₅₀, half-maximal inhibitory concentration; Mfs, myofibrillar proteins; O₂⁻, superoxide anion radical; •OH, hydroxyl radical; RCO, random-centroid optimization; RH, relative humidity; SOD, superoxide dismutase; SOSA, superoxide anion radical scavenging activity; U, unit

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Within the previous study (Nishimura and Saeki, 2016), antioxidant capacity against \( \cdot \text{OH} \) was used as an index of the antioxidant capability instead of SOSA, since SOSA in the context of a solid sample cannot be determined by electron spin resonance (ESR). Consequently, the antioxidant activity of optimized glycated chicken Mfs with maltose against \( \cdot \text{OH} \) (Nishimura and Saeki, 2016) could not be compared with other glycated Mfs, which were determined with SOSA studies (Nishimura et al., 2011a,b; Isono et al., 2012). Subsequently, in this study SOSA was used as an index of the antioxidant capability to determine the optimum preparative condition for functional glycated Mfs using RCO, and the antioxidant ability was compared to previously produced Mfs. Additionally, the differences in antioxidant activity between preparative conditions using monosaccharides and disaccharides are discussed.

### Materials and Methods

#### Materials and chemicals
Chicken breast meat was purchased from a local poultry farm immediately after slaughter. Biochemical-grade superoxide dismutase (SOD) from bovine erythrocytes was obtained from Sigma-Aldrich Co. (St. Louis, USA). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) (99.0%) was obtained from Labotec (Tokyo, Japan), and xanthine oxidase was purchased from Oriental Yeast Co. (Tokyo, Japan). All other chemicals were of reagent grade, and all other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan). Distilled water used in the ESR spectroscopy was pretreated with Chelex-100 resin (100–200 mesh; Bio-Rad, Hercules, USA).

#### Myofibrillar proteins (Mfs) preparation
Chicken Mfs were prepared following the method of Saeki (1997) with modifications. Chicken breast meat was ground with a food chopper and suspended in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.5). The solution was allowed to settle and the supernatant was decanted to waste. This step was repeated five times. The meat was then homogenized in a homogenizer (model AM-9; Nissei Co. Ltd., Tokyo, Japan) in 10 volumes (based on the initial muscle weight) of 0.1 M sodium phosphate buffer (pH 7.5) for 0.5 min at 10 000 rpm. This step was repeated four times. After passing through cotton gauze, 20% Triton X-100 solution was added to the filtrate to obtain a final concentration of 0.5% and stirred gently for 3 min. After standing for 10 min, the filtrate was centrifuged at 7 000 × g for 10 min to collect Mfs. The precipitate was resuspended in 50 mM NaCl and centrifuged. This procedure was performed five times. Purified chicken Mfs were obtained by filtering the solution through a nylon cloth. A portion of this fraction was dissolved in 0.5 M NaCl-15 mM sodium phosphate buffer (pH 7.5) for use as the native chicken Mfs solution. All preparation steps were carried out on ice.

#### Random-centroid optimization (RCO)
RCO (Nishimura et al., 1997, 1998; Goto et al., 2000; Nishimura et al., 2001; Goto et al., 2004; Nakai et al., 2009; Yan and Wang, 2015; Nishimura and Saeki, 2016) was used to determine the optimum preparative conditions for glycated chicken Mfs, meeting the requirements of greater than 60% solubility in a low ionic strength medium and the strongest antioxidant ability. Briefly, glycated chicken Mfs were prepared according to 13 vertices, which are a series of experimental conditions, dictating the four preparative condition parameters. Among glycated chicken Mfs, those with less than 60% solubility in a low ionic strength medium were evaluated as 100% in residual ratio of \( O_2^- \). Accordingly, the evaluation values of glycated chicken Mfs with greater than 60% solubility in a low ionic strength medium indicated figures below 100% because of the presence of antioxidative capacity. Vertices were evaluated to determine the smallest residual ratio of \( O_2^- \). Each vertex was assessed independently in triplicate or more. The experimental ranges of each factor were defined as follows: when maltose was used as the reaction sugar, temperature was 40–70°C, RH was 25–45%, reaction time was 24–48 h, and maltose mixing ratio (w/w) was 2–8. When ribose was used as the sugar, temperature was 30–45°C, RH was 35–45%, reaction time was 1–6 h, and ribose mixing ratio (w/w) was 8–14.

#### Glycation of chicken Mfs
The chicken Mfs were glycated following the method of Saeki (1997) with modifications. Chicken Mfs suspended in 50 mM NaCl were mixed with maltose or ribose at weight ratios determined by the RCO vertex. After adjusting the final protein concentration to 6.0 mg/mL, 5 mL of each Mfs-maltose or -ribose solution was transferred to a test tube (16 mm diameter), frozen at −80°C, and immediately lyophilized in a freeze-dryer (FDU-1110; Tokyo Rika Co., Ltd., Tokyo, Japan). This process destroyed the sarcomeres and exposed the Mfs. Lyophilization was terminated when the sample temperature reached 15–18°C. Each lyophilized protein powder was immediately stored at −40°C and used within 30 d of preparation.

The Maillard reaction was conducted by incubating the lyophilized powders under the 13 conditions defined by the RCO. An incubator/humidity cabinet (KCL-2000A; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) was used to control the temperature and RH.

#### Solubility of glycated chicken Mfs
The solubility of the glycated Mfs was measured according to the method of Saeki and Inoue (1997) with modifications. After glycation, the protein powder was immediately mixed with 0.1 M NaCl-15 mM sodium phosphate buffer (pH 7.5) in an T 10 basic ULTRA-TURRAX high-speed blender (IKA-Labotechnik, Staufen, Germany) at 13 500 rpm for 0.5 min, with a final protein concentration of 1.5 mg/mL. Homogenization was followed by centrifugation at 32 000 × g for 30 min at 4°C, and this step was repeated. The amounts of protein before centrifugation and in the supernatant were determined by the Kjeldahl method (AOAC, 1990). Total soluble Mfs were expressed as the percent protein concentration in the supernatant with respect to the total protein before
ESR Spectroscopy ESR spectroscopy was carried out with an X-band ESR spectrometer (JES-FA 100; Jeol Datum Ltd., Tokyo, Japan) at room temperature in a 0.4-mm flat cell (ES-LC 12; Jeol Datum Ltd.), according to the previously published method (Nishimura et al., 2011b). Manganese was used as an internal standard. ESR spectra were recorded under the following conditions: center field, 335.2 mT; sweep width, ±5 mT; microwave power, 4 mW; modulation frequency, 100 kHz; modulation width, 0.1 mT; amplitude, 200; sweep time, 2 min; and time constant, 0.1 s. A total of 32 µL of 0.78 unit (U)/mL xanthine oxidase was added as a reaction trigger to a mixture containing 10 µL of 50 mM diethylenetriamine pentaacetic acid, 75 µL of 99.5% dimethyl sulfoxide, 100 µL of 5 mM hypoxanthine, 25 µL of 9 M DMPO, 195.5 µL of sample solution, and 62.5 µL of 0.8 M NaCl-160 mM Tris-HCl (pH 7.5). After thorough mixing, the reaction mixture was transferred to a quartz cell. ESR spectrum measurement was started exactly 2 min after the addition of xanthine oxidase.

The comparative signal strength of O$_2^-$ induced by the hypoxanthine-xanthine oxidase system in the presence of glycated chicken Mfs was used for the evaluation of antioxidant capability, and the vertex that provided the smallest evaluation was sought. The ESR signal strength of O$_2^-$ induced by the hypoxanthine-xanthine oxidase system was regarded as 100%. When the solubility of glycated chicken Mfs with maltose did not surpass 60%, the evaluation (residual ratio of O$_2^-$) of this vertex was regarded as 100%, as described in the experiment for "RCO".

The authors have already confirmed the following phenomenon. When the concentration of DMPO increased, IC$_{50}$ increased proportionally, and the ratio of IC$_{50}$ to the concentration of DMPO was constant, suggesting that there was no inhibitory effect of glycated Mfs on xanthine oxidase.

Evaluation of the SOSA of SOD and glycated Mfs The SOSAs of SOD and the glycated Mfs were calculated as follows: O$_2^-$ was generated by the hypoxanthine-xanthine oxidase system, producing an ESR signal. ESR signal strength was converted to a signal intensity relative to that of manganese, which was defined as 100%. The comparative signal strengths in the presence of the glycated Mfs or SOD were calculated, and the relationship between the comparative signal strength and the concentration of the glycated proteins or SOD was graphed. The amount of glycated Mfs or the U of SOD that elicited 50% signal strength was considered as the IC$_{50}$ value of the glycated proteins or SOD. The IC$_{50}$ value of the glycated Mfs extract (mg/mL) was then converted into the SOSA (U/g of protein) based on the SOD IC$_{50}$ value (1.0 U/mL).

Protein determination The protein concentrations of dissolved and purified chicken Mfs were determined by following the methods of Kjeldahl (AOAC, 1990) and Lowry (Lowry et al., 1951), respectively, with ovalbumin as a standard. The biuret method (Gornall et al., 1949), which uses bovine serum albumin as a standard, was performed in other assays.

Statistical analysis Each experiment was performed on three different lots of glycated Mfs. The results are reported as mean values of at least three determinations, with the error bars indicating the standard deviation. Statistical analysis was performed using Microsoft Excel Ver. 2013 with Ekuseru-Tokei 2010 (Social Survey Research Information Co., Tokyo, Japan). P < 0.05 was considered significant.

Results and Discussion

Determination of the optimum preparative method of glycated chicken Mfs with maltose RCO was used to determine the optimal conditions for production of maltose-glycated chicken Mfs with the strongest antioxidant ability and greater than 60% solubility in a low ionic strength medium, varying temperature, RH, reaction time, and the maltose mixing ratio (w/w) (Table 1). Values of the parameters in each experiment were calculated using the RCO program, and then implemented for different preparations of glycated chicken Mfs. All data were mapped (Fig. 1). The mapping process aids in visualization of the experimental response surface, indicating the trend of the data (Nakai et al., 2009). Although RCO is usually repeated until an adequate response is achieved, in this study the approximate position of the optimum condition was clear after the first cycle of the RCO program. The best result was obtained at Vertex 12 with a temperature of 61°C, an RH of 38%, a reaction time of 33.9 h, and a maltose mixing ratio (w/w) of 5.59, as shown in Fig. 1.

When the index of antioxidant capability was •OH averting capacity, the optimum condition was a temperature of 57°C, an RH of 37%, a reaction time of 37.2 h, and a maltose mixing ratio (w/w) of 5.43 (Nishimura and Saeki, 2016). In this study, RH and maltose mixing ratio (w/w) were similar, the temperature was slightly higher, and the time was shorter. This subtle difference might be affected by the production yields of the intermediate and final Maillard reaction products with specific scavenging activity for O$_2^-$ or •OH.

Determination of the optimum preparative method of glycated chicken Mfs with ribose RCO was also used to determine the optimal conditions for production of ribose-glycated chicken Mfs with the strongest antioxidant ability and greater than 60% solubility in a low ionic strength medium (Table 2 and Fig. 2). The best response was obtained at Vertex 12 with a temperature of 38°C, an RH of 39%, a reaction time of 3.80 h, and ribose mixing ratio (w/w) of 10.4.

SOSA of the optimized glycated chicken Mfs Inhibitory effects of the optimized glycated chicken Mfs and native chicken Mfs on oxidation are shown in Fig. 3. Native chicken Mfs hardly demonstrated SOSA in the range of 2.0–6.0 mg of protein/mL, in agreement with our earlier studies focusing on glycated chicken Mfs with maltose (Nishimura et al., 2011b;
Table 1. Summary data for random-centroid optimization of SOSA using maltose.

<table>
<thead>
<tr>
<th>Vertex No.</th>
<th>Temperature (℃)</th>
<th>RH (%)</th>
<th>Reaction Time (h)</th>
<th>Maltose Mixing Ratio (w/w)</th>
<th>Evaluation (Residual ratio of $O_2^-$) (%)</th>
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<td>42</td>
<td>47.9</td>
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<td>37.9</td>
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</table>

a) When solubility of glycated Mfs with maltose did not exceed about 60%, the evaluation (residual ratio of $O_2^-$) of this vertex was regarded as 100%.
b) Re-centroid points of first cycle.

Fig. 1. Mapping results of experiments submitted by RCO for scavenging $O_2^-$ using maltose.

The comparative signal strength of $O_2^-$ induced by the hypoxanthine-xanthine oxidase system in the presence of glycated chicken Mfs was used for the evaluation of antioxidant capability, and the vertex that provided the smallest evaluation was sought. The ESR signal strength of $O_2^-$ induced by the hypoxanthine-xanthine oxidase system was regarded as 100%. When solubility of glycated chicken Mfs with maltose did not surpass 60%, the evaluation (residual ratio of $O_2^-$) of this vertex was regarded as 100%. (A) reaction temperature. (B) relative humidity. (C) reaction time. (D) maltose mixing ratio for Mfs (w/w). (●). Lines indicate probable trends. Arrow in each graph shows the best result.
Table 2. Summary data for random-centroid optimization of SOSA using ribose.

| Vertex No. | Temperature (°C) | RH (%) | Reaction Time (h) | Ribose Mixing Ratio (w/w) | Evaluation (Residual ratio of \( \text{O}_2 \)) (%)
<table>
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<td>11.2</td>
<td>57.8</td>
</tr>
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</table>

a) When solubility of glycated Mfs with ribose did not exceed about 60%, the evaluation (residual ratio of \( \text{O}_2 \)) of this vertex was regarded as 100%.

b) Re-centroid points of first cycle.

Fig. 2. Mapping results of experiments dictated by RCO for scavenging \( \text{O}_2 \) using ribose. Evaluation is the same as in Fig. 1. (A) reaction temperature. (B) relative humidity. (C) reaction time. (D) ribose mixing ratio for Mfs (w/w). (●). Lines indicate probable trends. Arrow in each graph shows the best result.
The antioxidant ability of native chicken Mfs (■), optimized glycated chicken Mfs with maltose (●) and optimized glycated chicken Mfs with ribose (▲) against O$_2$ was measured with ESR. The strength of O$_2$ generated by the hypoxanthine-xanthine oxidase system was converted to a signal intensity relative to that of manganese, which was regarded as 100%. Values are mean ± standard deviation (n ≥ 3).

Fig. 3. Antioxidant properties of glycated chicken Mfs

The antioxidant ability of native chicken Mfs (■), optimized glycated chicken Mfs with maltose (●) and optimized glycated chicken Mfs with ribose (▲) against O$_2$ was measured with ESR. The strength of O$_2$ generated by the hypoxanthine-xanthine oxidase system was converted to a signal intensity relative to that of manganese, which was regarded as 100%. Values are mean ± standard deviation (n ≥ 3).

Isono et al., 2012). Both optimized glycated Mfs produced using the data of Figs. 1 and 2 reduced the intensity of the O$_2$ signal in a concentration-dependent manner. The intensity of optimized glycated chicken Mfs with maltose and ribose eventually reached 25.8 ± 14.4% (n = 3) and 29.0 ± 7.3% (n = 3) when the protein concentration was 6.0 and 4.5 mg/mL, respectively. When both IC$_{50}$ values were converted into the SOSA based on the SOD IC$_{50}$ value (1.0 U/mL), they reached 274 ± 86 (n=3) and 368 ± 120 U/g of protein (n = 3), respectively. The SOSA of chicken Mfs glycated with maltose was 184 ± 40 U/g of protein (n = 3) when first discovered producing under limited conditions without using RCO (Nishimura et al., 2011b). This value increased about 1.5 times with Mfs produced using the RCO method. However, there was no significant difference between two SOSAs (274 ± 86 (n=3) and 184 ± 40 U/g of protein (n = 3)) of optimized maltose-glycated chicken Mfs and one produced without using the RCO program. In a previous study (Nishimura et al., 2011b), the glycation of chicken Mfs was conducted at a temperature of 60°C, an RH of 35%, a reaction time of 36 h, and a maltose mixing ratio (w/w) of 4. Meanwhile, the optimum condition was a temperature of 61°C, an RH of 38%, a reaction time of 33.9 h, and a maltose mixing ratio (w/w) of 5.59. Each condition was similar except for the maltose mixing ratio (w/w). This slight difference in each factor might induce production of Maillard reaction intermediates and final products with stronger antioxidative ability against O$_2$.

The glycation of chicken Mfs using maltose disaccharide required an incubation time of over 30 h, plus two days for removing free sugar through dialysis, to obtain the final glycated chicken Mfs. This suggests that maltose is unsuitable for practical use. Monosaccharides can undergo chemical reactions more easily; thus, we selected one as the sugar for glycation. Among monosaccharides, ribose has a five-member ring that should react faster than a six-member ring. As calculated using the inhibition curves shown in Fig. 3, SOSA in the optimized Mfs glycated with ribose showed 368 ± 120 U/g of protein (n = 3), which was approximately 1.3-fold higher than the optimized chicken Mfs glycated with maltose. In addition, the optimal condition for ribose glycation was a temperature of 38°C, an RH of 39%, a reaction time of 3.80 h, and a ribose mixing ratio (w/w) of 10.4. Compared to maltose, the reaction time was truncated by approximately 30 h. Moreover, the reaction temperature was lowered by approximately 20°C, although the ribose mixing ratio (w/w) increased by 1.7 times. Therefore, using ribose can effectively produce glycated chicken Mfs with the strongest antioxidant capability. However, the differences among these three SOSAs are not significant, suggesting that the antioxidant capability is controlled by the limit of greater than 60% solubility in a low ionic strength medium, despite the use of several types of sugar.

These results show that glycation using monosaccharides was practical. However, optimized chicken Mfs glycated with ribose could not form a gel by heating at 90°C (data not shown), unlike Mfs with maltose (Nishimura et al., 2015; Nishimura and Saeki, 2016), suggesting that optimized chicken Mfs glycated with ribose are not appropriate for use in processed food requiring gel-formation of Mfs, such as sausage. In a previous study using maltose (Nishimura and Saeki, 2016), heating at 90°C beyond 30 min prohibited the formation of a thermal gel, though 30 min of heat did produce a gel. This suggests the possibility that some kinds of intermediates and final products suppressed the formation of a thermal gel or dismantled the gel already formed. Accordingly, in the case of ribose, some similar intermediates that are quickly produced through the Maillard reaction might prohibit thermal gel formation. To confirm this system and obtain glycated chicken Mfs for practical use, glucose, a six-member ring monosaccharide, will be used instead. Six-member rings react with Mfs slowly, so that the process of acquiring antioxidant activity and losing heating gel formation capability can be more easily followed.

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