Chicken Ovotransferrin Variants $\text{OTF}^B$ and $\text{OTF}^C$ Harboring Substitution of GAT (Asp) to AAT (Asn) in the Codon 500 and their Antimicrobial Activity

Keiji Kinoshita¹, Si Lhyam Myint², Takeshi Shimogiri³, Hisham R. Ibrahim³, Kotaro Kawabe⁴, Shin Okamoto³, Yen-Pei Lee⁵, Yoichi Matsuda¹ and Yoshizane Maeda³

¹Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
²United-graduate School of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
³Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
⁴Natural Science Centre for Research and Education, Kagoshima University, Kagoshima 890-0065, Japan
⁵Faculty of Animal Science, National Chung Hsing University, 250 Kuo-Kuang Road, Taichung 402, Taiwan

Chicken egg white ovotransferrin (OTF) has been reported to exist in three electrophoretic variants (OTF$^A$, OTF$^B$ and OTF$^C$). In this report, we identified a causal polymorphism between the OTF$^B$ and OTF$^C$ variants in Japanese and Taiwanese native chickens and compared the antibacterial activity between these two variants. The cDNA sequence analyses from Satsumadori oviducts revealed that three non-synonymous SNPs T1809G (Ser52Ala), A2258G (Ile96Val) and G7823A (Asp500Asn) corresponded to the OTF electrophoretic phenotypes. Of the three SNPs, the G7823A mutation perfectly corresponded to the electrophoretic phenotypes OTF$^B$ (G/G, Asp500Asp), OTF$^B/C$ (G/A, Asp500Asn) and OTF$^C$ (A/A, Asn500Asn) in three chicken populations. The variants OTF$^B$ and OTF$^C$ exhibited similar antibacterial potency against Gram-positive and Gram-negative bacteria. This study provides, for the first time, molecular information on polymorphism of OTF$^B$ and OTF$^C$ variants of chicken ovotransferrin and its effect on the antimicrobial activity of the respective variants.

Key words: antibacterial activity, chicken egg white, electrophoretic variant, ovotransferrin, SNP

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Introduction

Transferrin (TF) is an iron-binding glycoprotein mainly present in serum and egg white. Egg white TF is called ovotransferrin (OTF) or conalbumin. OTF is synthesized in the oviduct and secreted into the egg white (Stevens, 1991). OTF constitutes the second major protein (12% of the total proteins) in hen egg white. OTF plays a key role in the antibacterial activity of egg albumen (Valenti et al., 1983; Ibrahim et al., 1998, 2000; Baron et al., 1999) and exerts antiviral activity towards the Marek’s disease virus (MDV) (Giansanti et al., 2002, 2005).

In chickens, three OTF variants (OTF$^A$, OTF$^B$ and OTF$^C$) have been demonstrated by starch or polyacrylamide gel electrophoresis and are believed to be controlled by three alleles ($OTF^A$, OTF$^B$ and OTF$^C$) at a single OTF locus (Baker, 1968; Baker et al., 1970). The OTF$^B$ allele and is commonly found in many improved breeds and native chicken populations (Kinoshita et al., 2002). The OTF$^A$ and OTF$^C$ alleles were observed at very low frequencies in limited improved chicken populations and Asian native populations. In our previous study, the allele frequencies of $OTF^A$, OTF$^B$ and OTF$^C$ in 27 populations including improved breeds and Asian native populations were observed in the range of 0.00–0.18, 0.73–1.00 and 0.00–0.23, respectively (Kinoshita et al., 2002). In addition, OTF$^A$ variant was not found in seven Japanese chicken populations (Myint et al., 2010). Although the electrophoretic variants have been thought to arise from the three OTF alleles, the difference of these variants on the primary structure of the protein and the antibacterial activity is yet to be unraveled. The objective of this study is to examine the contribution of the two OTF variants (OTF$^B$ and OTF$^C$) to the different electrophoretic mobility (surface charge) and their effect on antibacterial activity.
Materials and Methods

Ethics Statement
Animal care and all experimental procedures were approved by the Animal Experiment Committee, Graduate School of Bioagricultural Sciences, Nagoya University (approval no 2014021202), and the experiments were conducted according to Regulations on Animal Experiments at Nagoya University.

Egg White Samples
A total of 395 fresh egg white samples were collected from two Japanese natives (Satsumadori, n = 155; Ehime-jidori, n = 50), three Taiwanese natives (Tsin-Yi n = 35, Hua-Tung n = 21 and Ju-Chi n = 34) and two commercial populations (White egg layer n = 50 and Brown egg layer n = 50). One Satsumadori population (n = 48) and Ehime-jidori were kept in Kagoshima University (KU). Three Taiwanese natives were kept in Chung Hsing University. The other Satsumadori (n = 107) and two commercial populations were kindly provided from Kagoshima Prefectural Institute for Agricultural Development (KPI). Ehime-jidori is a Japanese native chicken that is newly found in the southwest region of Ehime Prefecture around the 1990s (Tanabe et al., 2000).

Screening of the Chicken OTF Electrophoretic Variants
Chicken OTF variants were examined by using 8% non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) (Davis 1964) as described below. The egg white samples were diluted 8 times by volume with dilution buffer [2 ml of 0.5 M Tris-HCl (pH 6.8), 1.6 ml glycerol, and 0.4 ml of 0.05% (wt/vol) bromophenol blue] and electrophoresis was conducted at 3.5 mA/cm constant current in 8% Native-PAGE with Tris/glycine buffer [25 mM Tris base, 192 mM glycine (pH 8.3)]. Each gel slab was stained with 0.125% (wt/vol) coomassie brilliant blue R-250 (CBB-R250) in glycerol, and 0.4 mL of 0.5% (wt/vol) bromophenol blue and electrophoresis was conducted at 3.5 mA/cm constant current in 8% Native-PAGE with Tris/glycine buffer [25 mM Tris base, 192 mM glycine (pH 8.3)]. Each gel slab was stained with 0.125% (wt/vol) coomassie brilliant blue R-250 (CBB-R250) in methanol-acetic acid-water (40:7:53), destained in methanol-acetic acid-water (25:10:65) and the OTF phenotypes judged based on mobility shift of OTF bands.

Total RNA Extraction from Oviduct and cDNA Synthesis, OTF cDNA Cloning
Oviduct tissues were collected from three egg-laying Satsumadori hens identified as the electrophoretic phenotypes OTFB, OTFB/C and OTFC. Total RNA was extracted with TRIzol reagent, following manufacturer instructions (Life Technologies, Carlsbad, CA, USA). One μg of RNA was then incubated with 50 pmol of oligo dT primers for 10 minutes at 65°C, and then subjected to a reverse transcriptase (RT) reaction. The final reaction volume was 20 μl, containing 1×RT buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 5 mM MgCl2, 10 mM dithiothreitol, 500 μM of each dNTP, 40 units of RNaseOUT (Life Technologies Japan Ltd.) and 200 units of SuperScript II RNase H Reverse Transcriptase (Life Technologies). Oviduct cDNAs were synthesized at 37°C for 50 min following inactivation of SuperScript II by heating at 70°C for 15 min. To remove template mRNAs, samples were treated with 1 unit of RNaseH at 37°C for 20 min.

On the basis of the sequence of the chicken OTF gene (accession no. Y00407) available in the NCBI database, three pairs of PCR primers were designed (Table 1), enabling the amplification of the chicken OTF cDNA. Three overlapping fragments of the OTF cDNA for each phenotype OTFB, OTFB/C and OTFC of Satsumadori were amplified and directly sequenced using primers that are shown in Table 1. The PCR mixture contained 40 ng of oviduct cDNA, PCR buffer containing 2 mM MgCl2, 200 μM of each dNTP, 10 μM each of forward and reverse primers and 1 unit of Ex Taq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) in a final volume of 20 μl. The PCR reactions were performed on a GeneAmp PCR system 9700 (Life Technologies) with the following profile: initial denaturation of 3 min at 94°C, 35 cycles of 94°C for 30 s, 60–62°C for 30 s, and 72°C for 1 min, and a final elongation step of 7 min at 72°C.

Blood Samples and Genomic DNA Extraction
A total of 29 whole blood samples were aseptically taken from the wing veins of hens. These hens consisted of 12 Satsumadori (3 OTFB, 8 OTFB/C and 1 OTFC), three Ehime-jidori (3 OTFB), and 14 Ju-Chi (5 OTFB and 9 OTFB/C). Genomic DNAs were extracted with PUREGENE DNA isolation kit (Genta Systems, Inc., MN, USA). The purified genomic DNA was used as a template for PCR amplification (exons 1–5 and exons 10–17) of the OTF gene as described below.

PCR Amplification using Genomic DNA
Two segments (3,508 bp of exon 1–5 and 3,960 bp of exon 10–17) of the chicken OTF gene were amplified by using two pairs of primers (TF1 with TF2 and TF5 with TF6) and directly sequenced using three primers (TFex2F, TFint3R and TFint12F) that are shown in Table 1. The sequence of these primers was also adopted from the nucleotide sequence of the chicken OTF gene (NCBI accession no. Y00407). These regions correspond to exons 1–5 and exons 10–17 of the chicken OTF gene. PCR reactions were carried out in a final volume of 50 μl containing 100 ng genomic DNA, 2.5 units of Ex Taq polymerase (TaKaRa Bio Inc.), 1× reaction buffer (supplied with Ex Taq) with 2 mM MgCl2, 200 μM of each dNTP and 10 μM of each primer. The reactions were carried out in a GeneAmp PCR System 9700 with the following profile: initial denaturation of 3 min at 94°C; 35 cycles of 94°C for 30 sec, 60–65°C for 30 sec, and 72°C for 5 min; and final elongation step of 7 min at 72°C.

DNA Sequencing and Mutation Detection
Following the conditions mentioned above, PCR fragments of chicken OTF gene were amplified. After electrophoresis, targets were excised from the agarose gel and purified with Gel-M gel extraction kit (Viogene BioTek, New Taipei City, Taiwan). These purified samples were used as templates for direct sequencing. DNA sequencing reactions were carried out using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Life Technologies) as recommended by the manufacturer. Briefly, the reaction contained 0.2 μl of PCR product and 3.2 pmol of either the forward or reverse primers that are shown in Table 1. Sequence analysis was performed by using an ABI PRISM 3100 Genetic Analyzer (Life Tech-
Isolation of Ovotransferrin from Chicken Egg White

HCl. The solution was stirred overnight at 2 °C. Distilled water and the mixture was adjusted to pH 6 with 1 N HCl. Briefly, egg white was diluted with 3 volumes of distilled water and lyophilized. To examine the purity and approximate molecular mass of the purified ovotransferrin, the transferrin peak was finally desalted by dialysis against the same column with a shallow gradient. The collected ovotransferrin peak was re-chromatographed on the column with an on-line BioFrac Fraction Collector (BioLogic LP, low-pressure chromatography system, BioSepra, Cergy-Saint-Christophe, France) that was connected to a Cergy-Saint-Christophe, France) that was connected to a BioLogic LP low-pressure chromatography system (Bio-Rad, Hercules, CA, USA). Protein was eluted with a linear gradient of 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5), and monitored at 280 nm. Peaks were collected automatically using an on-line BioFrac Fraction Collector (Bio-Rad). Ovotransferrin peak was re-chromatographed on the same column with a shallow gradient. The collected ovotransferrin peak was finally desalted by dialysis against distilled water and lyophilized. To examine the purity and approximate molecular mass of the purified ovotransferrin, SDS-PAGE was conducted in accordance with the method of Laemmli (1970). Samples were run on a 12% polyacrylamide gel in the presence of 0.1% SDS for 3 h at 20 mA. A protein molecular mass marker (LMW-SDS Marker Kit (14,000–97,000), Nippon Genetics, Tokyo, Japan) was used. Protein bands were visualized by staining with CBB R-250.

Antibacterial Assay

Antibacterial assay was performed according to our previous report (Myint et al., 2012). Samples of purified chicken egg white ovotransferrin with the phenotypes OTFA and OTFB were used for antibacterial assay. We tested the antibacterial activity of the samples against two bacterial strains: Staphylococcus aureus (Gram-positive) and Escherichia coli (XL-1 blue; Gram-negative). Bacteria were grown overnight in brain heart infusion broth (BHI; Nissui, Tokyo, Japan) at 37 °C. Bacterial cells were suspended in trypticase soy broth (TSB; Nissui, Tokyo, Japan) to give 2 × 10^7 colony forming units (cfu)/ml (Ibrahim et al., 1996). Two-fold dilutions of ovotransferrin (1 mg/ml initial concentration) in 1× PBS buffer were prepared in each well of a 96-well microtiter plate and mixed with an equal volume of bacterial suspension in TSB broth to give the required range of concentrations (3.9–250 μg/ml). Controls were incubated with PBS instead of diluted ovotransferrin. The cultures in the microtiter plates were incubated at 37 °C for 16 h, during which period the kinetics of inhibition of growth of the test organism was monitored spectrophotometrically every two hours at 620 nm using a Mithras LB940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). All assays were conducted in three experiments performed in duplicate. Bacterial growth at a given time was represented as the average change (ΔOD620) in optical density, using the formula: ΔOD620 = OD620 at a given time – OD620 at time zero. The bacterial growth values were subjected to repeated measures analysis of variance (ANOVA) using a general linear model (GLM), with variant as a between subjects variable and incubation time as a within subjects variable. If a significant interaction was detected between a variant and incubation time, significant differences between groups for each time point were detected by using Tukey’s HSD (Honestly Significant Difference) test. The statistical analysis was performed in the SAS software (SAS Inst. Inc., Cary, NC).

| Table 1. Oligonucleotide primers used in this study |
|------------------------|--------------------------|--------------------------|------------|
| Primer | Primer sequence (5’ to 3’ ) | Nucleotide position | PCR product (bp) |
| PCR amplification and sequencing |
| TF1 (fwd)2 | 5‘- GACGGTCTGTGACCAACCGCTG -3’ | 284–307 | 720 or 3508 |
| TF2 (rev)2 | 5‘- CTTGACCTGAGTACGTTCGGAG -3’ | 3792–3769 | 720 or 3508 |
| TF3 (fwd) | 5‘- GTCCAAAACAGCTACTAAGTGTG -3’ | 3275–3298 | 1026 |
| TF4 (rev.) | 5‘- CTTGACCTGAGTACGTTCGGAG -3’ | 7034–7011 | 1026 |
| TF5 (fwd)2 | 5‘- AAAGGTAAAGGCAAGATGCTGTGAGC -3’ | 6690–6713 | 906 or 3960 |
| TF6 (rev)2 | 5‘- CACGAGAGGGATCATGCGCGAAG -3’ | 10709–10686 | 906 or 3960 |
| cDNA sequencing |
| TFexSF (fwd) | 5‘- AGTGACCTTTGGCGTGACCAACAG -3’ | 5018–5040 | 5214–5181 |
| TFexSR (rev.) | 5‘- CTTTTCGGCATGCTCTGGATGCC -3’ | 5181–5179 | |
| Genomic sequencing |
| TFex2F (fwd) | 5‘- CAAGTCAGTCATCAGATGGTG -3’ | 1721–1741 | |
| TFint3R (rev.) | 5‘- TGGAAAGCTCCAGAATATCCAGAA -3’ | 3792–3769 | |
| TFint12F (fwd) | 5‘- TATGCTGACAGTAGGCACTCCTG -3’ | 7569–7592 | |

1 Nucleotide positions are defined according to the chicken OTF gene (NCBI accession number Y00407).
2 Two primer pairs were used for amplification of cDNA and genomic DNA of the OTF gene.
Results

Electrophoretic Polymorphism of the OTF Protein

Table 2 shows the distributions of electrophoretic phenotypes and gene frequencies of OTF in eight chicken populations. Three OTF phenotypes (OTF<sup>B</sup>, OTF<sup>B/C</sup> and OTF<sup>C</sup>) were observed in two Satsumadori, Hua-Tung and Ju-Chi populations. The allele frequencies of OTF<sup>C</sup> were 0.104, 0.238, 0.048 and 0.162 in the four populations, respectively. The OTF<sup>B</sup> allele was fixed in two natives (Ehime-jidori and Tsin-Yi) and two commercial chickens. The OTF<sup>A</sup> allele was not detected in this study.

Comparison of the OTF cDNA Sequences for the Three Phenotypes

The OTF cDNA sequences were obtained from oviduct RNAs of three Satsumadori hens with the phenotypes OTF<sup>B</sup>, OTF<sup>B/C</sup> and OTF<sup>C</sup>. The OTF cDNA sequences for the phenotypes OTF<sup>B</sup>, OTF<sup>B/C</sup> and OTF<sup>C</sup> were deposited in the DDBJ database under accession numbers AB215094, AB222603 and AB215095, respectively. Table 3 showed the nucleotide and deduced amino acid substitutions in three phenotypes from comparison with chicken OTF gene sequence (NCBI accession no. Y00407). Comparison of the protein coding sequences of the three OTF phenotypes revealed seven coding single nucleotide polymorphisms (cSNPs). Out of the seven, three cSNPs: T1809→G changing codon 52 from TCA to GCA (Ser→Ala) in exon 2, A2258→G changing codon 96 from ATT to GTT (Ile→Val) in exon 3, and G7823→A changing codon 500 from GAT to GAT (Asp→Asn) in exon 12, corresponded to the electrophoretic phenotypes. In addition to these seven cSNPs, from the sequence comparisons with Y00407, we found two non-synonymous sites C3412→A and G10171→A in exon 4 and exon 16 resulting in Leu152→Ile and Ser686→Asn, respectively.

Identification of Causal Polymorphism in the Three Non-synonymous cSNPs

To further confirm whether the three non-synonymous cSNPs are causal between OTF<sup>B</sup> and OTF<sup>C</sup> variants, additional 29 hens, which had the OTF electrophoretic phenotypes, were selected from Satsumadori, Ehime-jidori and Ju-Chi populations and were genotyped for these cSNPs. These results revealed that the two cSNPs at nucleotide position 1,809 and 2,258 were found in the same phenotype, but the G to A substitution at position 7,823 in exon 12 was the only difference among the phenotypes OTF<sup>B</sup>, OTF<sup>B/C</sup> and OTF<sup>C</sup>.

### Table 2. Distribution of electrophoretic phenotypes and allele frequencies of OTF in eight chicken populations

| Population     | Number of samples | OTF<sup>B</sup> | Phenotype OTF<sup>B/C</sup> | OTF<sup>C</sup> | Allele frequency |
|----------------|-------------------|----------------|-----------------------------|----------------|-----------------|
| **Japanese native breeds** |                   |                |                             |                |                 |
| Satsumadori (KU) | 48                | 40             | 6                           | 2              | 0.896 0.104     |
| Satsumadori (KPI) | 107               | 64             | 35                          | 8              | 0.762 0.238     |
| Ehime-jidori | 50                | 50             | 0                           | 0              | 1.000 0.000     |
| **Taiwanese native breeds** |                 |                |                             |                |                 |
| Tsin-Yi | 35                | 35             | 0                           | 0              | 1.000 0.000     |
| Hua-Tung | 21                | 19             | 2                           | 0              | 0.952 0.048     |
| Ju-Chi | 34                | 23             | 11                          | 0              | 0.838 0.162     |
| **Commercial breeds** |               |                |                             |                |                 |
| White egg layer | 50                | 50             | 0                           | 0              | 1.000 0.000     |
| Brown egg layer | 50                | 50             | 0                           | 0              | 1.000 0.000     |

### Table 3. Nucleotide and deduced amino acid variations in the chicken OTF gene detected in Satsumadori hens

| Nucleotide position (amino acid position) | Y00407 |
|------------------------------------------|--------|
| T 1809                                   | A 2258 |
| (Ser52)                                  | (Ile96) |
| (Thr132)                                 | (Leu152) |
| (Pro237)                                 | (Ile364) |
| (Asp500)                                 | (Gly510) |
| (Ser686)                                 |        |
| OTF<sup>B</sup>                          | G      |
| T (Ser)                                  | A      |
| (Ser/Ala)                                | A      |
| A/G                                      | C      |
| A (Ile)                                  | A (Ile) |
| A (Ile)                                  | G      |
| A (Ile)                                  | G/C    |
| C/T                                      | G      |
| (Asp)                                    | G/A    |
| A (Asn)                                  |        |
| OTF<sup>B/C</sup>                        | G      |
| A/G                                      | C/G    |
| A (Ile)                                  | A (Ile) |
| A (Ile)                                  | G/A    |
| A (Ile)                                  | C/T    |
| G/A                                      | G/A    |
| A (Asn)                                  | A (Asn) |
| OTF<sup>C</sup>                          | A      |
| G (Ala)                                  | G (Val) |
| G (Val)                                  | C      |
| C/G                                      | A      |
| A (Ile)                                  | G/A    |
| A (Ile)                                  | C/T    |
| A (Asn)                                  | G/A    |
| A (Asn)                                  |        |
| Amino acid change                        | Ser>Ala|
| Exon 2                                   | Ile>Val|
| Thr                                      | Leu>Ile|
| Pro                                      | Ile    |
| Asp>Asn                                  | Gly    |
| Exon 12                                  |        |
| Exon 13                                  |        |
| Exon 16                                  |        |

Nucleotide positions are defined according to the chicken ovotransferrin gene (NCBI accession number Y00407). Bold letters indicate the non-synonymous substitutions. Italic letters indicate synonymous substitutions.
Table 4. Correspondence of three cSNP genotypes to the OTF electrophoretic phenotypes

| Phenotype | Number of samples | T1809G (Ser52Ala) | A2258G (Ile96Val) | G7823A (Asp500Asn) |
|-----------|------------------|------------------|------------------|------------------|
| OTF<sup>B</sup> | 11 | 8 | 3 | 0 | 11 | 0 | 0 | 11 | 0 | 0 |
| OTF<sup>B/C</sup> | 17 | 0 | 15 | 2 | 7 | 10 | 0 | 0 | 17 | 0 |
| OTF<sup>C</sup> | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 |

Table 5. Antibacterial effects of the purified OTF<sup>B</sup> and OTF<sup>C</sup> variants against <i>S. aureus</i> and <i>E. coli</i>

| Time | S. aureus | E. coli |
|------|----------|---------|
|      | Control<sup>1</sup> | OTF<sup>B</sup> | OTF<sup>C</sup> | Control<sup>1</sup> | OTF<sup>B</sup> | OTF<sup>C</sup> |
| 0 h  | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 | 0.000±0.000 |
| 2 h  | 0.032±0.000<sup>a</sup> | 0.008±0.002<sup>b</sup> | 0.011±0.003<sup>b</sup> | 0.018±0.009<sup>a</sup> | 0.003±0.001<sup>b</sup> | 0.008±0.002<sup>a</sup> |
| 4 h  | 0.029±0.002<sup>a</sup> | 0.022±0.001<sup>b</sup> | 0.021±0.001<sup>b</sup> | 0.023±0.000<sup>a</sup> | 0.015±0.001<sup>b</sup> | 0.017±0.001<sup>b</sup> |
| 6 h  | 0.060±0.000<sup>a</sup> | 0.051±0.005<sup>b</sup> | 0.051±0.004<sup>b</sup> | 0.044±0.002<sup>a</sup> | 0.035±0.003<sup>b</sup> | 0.034±0.003<sup>b</sup> |
| 8 h  | 0.108±0.000<sup>a</sup> | 0.088±0.005<sup>b</sup> | 0.090±0.002<sup>b</sup> | 0.080±0.001<sup>a</sup> | 0.064±0.002<sup>b</sup> | 0.065±0.002<sup>b</sup> |
| 10 h | 0.130±0.002<sup>a</sup> | 0.107±0.005<sup>b</sup> | 0.111±0.002<sup>b</sup> | 0.086±0.002<sup>a</sup> | 0.070±0.000<sup>b</sup> | 0.073±0.001<sup>b</sup> |
| 12 h | 0.140±0.001<sup>a</sup> | 0.119±0.004<sup>b</sup> | 0.120±0.002<sup>b</sup> | 0.099±0.001<sup>a</sup> | 0.079±0.001<sup>b</sup> | 0.082±0.002<sup>b</sup> |
| 16 h | 0.185±0.001<sup>a</sup> | 0.139±0.005<sup>b</sup> | 0.142±0.002<sup>b</sup> | 0.131±0.006<sup>a</sup> | 0.098±0.004<sup>b</sup> | 0.096±0.002<sup>b</sup> |

<sup>a,b</sup> Means (±SE) within a time and bacteria not sharing a common superscript differ (<i>P</i>&#x2260;0.05). Values indicate the absorbance (A620nm) of the bacterial culture at the given time.

<sup>1</sup> Control was incubated with PBS instead of the purified ovotransferrin.

and OTF<sup>C</sup> and agreed well with the phenotypes OTF<sup>B</sup> (G/G, Asp500Asp), OTF<sup>B/C</sup> (G/A, Asp500Asn) and OTF<sup>C</sup> (A/A, Asn500Asn) (Table 4).

**Effect of the OTF Variants on the Antibacterial Activity**

The OTF<sup>B</sup> and OTF<sup>C</sup> variants were purified from egg whites of the homozygous chickens to make results of antibacterial assay clear. SDS-PAGE showed that both of the purified proteins appeared as a single band that was approximately equal in size to 78 kDa. A broth microdilution method was used in this study to determine the antibacterial activity of the purified chicken ovotransferrin variants. A Gram-positive (<i>S. aureus</i>) and Gram-negative (<i>E. coli</i>) bacteria were used as test strains. For clarity of data presentation, the antibacterial activity of the purified ovotransferrin is presented at a concentration of 31.25 μg/ml. As shown in Table 5, the turbidity of the control bacterial cultures increased with incubation time for both types of bacteria. The addition of different variants of the purified ovotransferrin resulted in a decrease in the growth of both <i>S. aureus</i> and <i>E. coli</i>, indicating growth inhibition. The mean (±SE) bacterial growth of the OTF<sup>B</sup> and OTF<sup>C</sup> variants and control are shown in Table 5. Repeated measures ANOVA revealed that there was no significant interaction between variant and time in both bacteria (<i>P</i>&#x2260;0.01 in <i>S. aureus</i> and <i>P</i>&#x2260;0.05 in <i>E. coli</i>). Thus, we analyzed by Tukey’s HSD test at each time point in each bacteria. The results showed that significant differences between the variants and control were found from 2 h after incubation (<i>P</i>&#x2260;0.05 in <i>S. aureus</i> and <i>P</i>&#x2260;0.01 in <i>E. coli</i>), suggesting that both purified ovotransferrin inhibited bacteria growth. However, no significant differences were found between the two variants in both bacteria (<i>P</i>&#x2260;0.05).

**Discussion**

This study investigated the relationship between non-synonymous polymorphisms in the <i>OTF</i> gene and electrophoretic OTF variants and a difference among the variants for antibacterial activity. We focused on the difference between the variants OTF<sup>B</sup> and OTF<sup>C</sup>. This was because fresh eggs were necessary for antibacterial assay and no bird with OTF<sup>A</sup> was obtained in seven Japanese populations (Myint et al., 2010). In addition, we used the homozygous chickens for antibacterial assay to make the results clear. It was thought to be difficult for us to obtain both the OTF<sup>A</sup> and OTF<sup>C</sup> homozygous chickens because of low allelic frequencies. The genetic study for the OTF<sup>A</sup> variant needs to be conducted in the near future.

In this study, we identified one cSNP (G7823A) corresponding to the OTF phenotypes. This SNP was a non-synonymous substitution that results in conversion of aspartic acid (GAT; allele OTF<sup>B</sup>) to asparagine (AAT; allele OTF<sup>C</sup>) at amino acid residue 500. It has been reported that the difference in electrophoretic mobility is attributed mainly to the net surface charge of a protein, and the ratio of detectable amino acid substitution is estimated to be about 25% of the total amino acid substitution (Nei and Chakraborty, 1973). Thus, although conversion of the negatively charged aspartic acid to asparagine at residue 500 decreases the negative charge of the mature protein in the OTF<sup>C</sup> variant, the other two non-charged amino acid sub-
stitutions (Ser52Ala, Ile96Val) had no effect on the protein net surface charge of both variants: These two constitute the polymorphisms among individuals, breeds or strains. Ovotransferrin is a key antimicrobial protein in avian egg albumen (Ibrahim et al., 1998, 2000). Therefore, it was necessary to evaluate the effect of these variants of ovotransferrin on antibacterial activity. Although the purified OTF variants exhibited considerable growth inhibition of both test strains, there was no statistical significant effect between the two OTF variants in this study. These results suggest that these variants had negligible effect on the antibacterial activity.

The position and chemistry of an amino acid residue affects the structure and biological function of a protein as well as its stability. Ibrahim et al. (1998, 2000) reported the presence of the major antimicrobial peptide within the amino acid sequence from 109 to 200 of ovotransferrin. Three mutations related to the OTF variants in Satsumadori, which are distant from the antibacterial peptide region, were as expected would not influence the antibacterial activity. Ibrahim et al. (2006) reported that OTF underwent self-cleavage thus produced two disulfide kringles peptides (residues 115–211 and 454–544). These peptides of OTF were reported to possess SOD-like superoxide anion scavenging activity and anticancer activity against human colon and breast cancer cells (Ibrahim et al., 2007; Ibrahim and Kiyono, 2009). The Asp500Asn mutation lies in the kingle peptide of C-lobe (454–544), which increases surface positive charges thus may affect anticancer action of OTF. This would merit further investigation.

Serum TF and egg white OTF are translated by same gene because they have the same amino acid sequence and parallel electrophoretic phenotype (Baker et al., 1970). It is known that both proteins are only different in glycosylated form (Williams 1962, 1968; Jacquinot et al., 1994). The major role of TF is iron transport to other tissues (Keung and Azari, 1982; Oratore et al., 1990). In addition, it is believed that TF plays an important role in developing nervous system and bone formation during embryogenesis (Oh et al., 1986). Examination of the effect of these substitutions on the other functions of TF would merit further investigations.

In this study, non-synonymous substitutions were found, which were classified as the same OTF phenotype. The Ser52Ala and Ile96Val mutations were novel amino acid substitutions. Another mutation (Ile152Leu) was found by comparison of the coding sequences of three Satsumadori hens and database sequences (Y00407). This Ile152Leu mutation may influence the antibacterial activity because the mutation is located within the reported antibacterial peptide region.

In conclusion, we identified a non-synonymous SNP (G→A) at position 7,823 in exon 12 corresponding to the electrophoretic variants. This substitution converts the aspartic acid (Asp) for OTFB variant at amino acid residue 500 to asparagine (Asn) in the OTFC variant. The replacement of Asp500 with Asn500 reflects the difference in electrophoretic mobility between the OTFB and OTFC variants as a consequence of change in net surface charge of the gene products of two alleles, but had no significant effect on the antimicrobial activity. Further research will be required to examine whether these cSNPs have contribution to the other functions of OTF.

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