JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0160055

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

Keiji Kinoshita[1], Si Lhyam Myint[2], Takeshi Shimogiri[3], Hisham R. Ibrahim[3], Kotaro Kawabe[4], Shin Okamoto[3], Yen-Pei Lee[5], Yoichi Matsuda[1] and Yoshizane Maeda[3]

[1] Avian Bioscience Research Center, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464−8601, Japan
[2] United-graduate School of Agriculture, Kagoshima University, Kagoshima 890−0065, Japan
[3] Faculty of Agriculture, Kagoshima University, Kagoshima 890−0065, Japan
[4] Natural Science Centre for Research and Education, Kagoshima University, Kagoshima 890−0065, Japan
[5] Faculty 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

J. Poult. Sci., 53: 257−263, 2016

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* is a major 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.

Received: April 6, 2016, Accepted: May 9, 2016
Released Online Advance Publication: June 25, 2016
Correspondence: T. Shimogiri, Kagoshima University, Korimoto, Kagoshima 890−0065, Japan. (E-mail: simogiri@agri.kagoshima-u.ac.jp)

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% nondenaturing polyacrylamide gel electrophoresis (Native-PAGE) (Davis 1964) as described below. The egg white samples were diluted 8 times by volume with dilution buffer [2 m*l* of 0.5 M Tris-HCl (pH 6.8), 1.6 m*l* glycerol, and 0.4 m*l* 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 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 OTF^{B}, $OTF^{B/C}$ and OTF^{C}. Total RNA was extracted with TRIzol reagent, following manufacturer instructions (Life Technologies, Carlsbad, CA, USA). One μg of RNA was then incubated with 50 pmoles of oligo dT primers for 10 minutes at 65℃, 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 $MgCl_2$, 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℃ for 50 min following inactivation of SuperScript II by heating at 70℃ for 15 min. To remove template mRNAs, samples were treated with 1 unit of RNaseH at 37℃ 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 OTF^{B}, $OTF^{B/C}$ and OTF^{C} 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 $MgCl_2$, 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℃, 35 cycles of 94℃ for 30 s, 60−62℃ for 30 s, and 72℃ for 1 min, and a final elongation step of 7 min at 72℃.

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 OTF^{B}, 8 $OTF^{B/C}$ and 1 OTF^{C}), three Ehime-jidori (3 OTF^{B}), and 14 Ju-Chi (5 OTF^{B} and 9 $OTF^{B/C}$). Genomic DNAs were extracted with PUREGENE DNA isolation kit (Gentra 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 $MgCl_2$, 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℃; 35 cycles of 94℃ for 30 sec, 60−65℃ for 30 sec, and 72℃ for 5 min; and final elongation step of 7 min at 72℃.

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 μg of PCR product and 3.2 pmoles 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-

Table 1. **Oligonucleotide primers used in this study**

Primer	Primer sequence (5′ to 3′)	Nucleotide position[1]	PCR product (bp)
PCR amplification and sequencing			
TF1 (fwd)[2]	5′ - GACGGTCTGTGACCAACACCGCTG -3′	284–307	720 or 3508
TF2 (rev.)[2]	5′ - TGGAAAGCTCCAGAATATCCAGAA -3′	3792–3769	720 or 3508
TF3 (fwd)	5′ - GCTCCACAACCAGCTACTATGCTG -3′	3275–3298	1026
TF4 (rev.)	5′ - CTGGTCGTTCATCTGTTTTGCTGC -3′	7034–7011	1026
TF5 (fwd)[2]	5′ - AAAGGTGAAGCAGATGCTGTTGCC -3′	6690–6713	906 or 3960
TF6 (rev.)[2]	5′ - CACGAGAGGAGTCATGGCGCGAAG -3′	10709–10686	906 or 3960
cDNA sequencing			
TFex8F (fwd)	5′ - AGTGACTTTGGCGTGGACACCAAG -3′	5018–5040	
TFex8R (rev.)	5′ - CTTTTCCGCATGCTCTGGATGGCAC -3′	5214–5181	
Genomic sequencing			
TFex2F (fwd)	5′ - CAAGTCAGTCATCAGATGGTG -3′	1721–1741	
TFint3R (rev.)	5′ - CTCTCATATCTGCCGCTCATTCAG -3′	2328–2351	
TFint12F (fwd)	5′ - TATGCTGCACGTAGATGCCCTCTG -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.

nologies) and sequence alignment with the chicken *OTF* gene sequence (accession no. Y00407) available in the NCBI database was obtained by using CLUSTALW (http://clustalw.genome.ad.jp/) (Thompson *et al*., 1994).

Isolation of Ovotransferrin from Chicken Egg White

Ovotransferrin was purified from fresh egg white of the respective mutant chickens (one OTF^B and one OTF^C) through two successive steps on cation exchange chromatography. Briefly, egg white was diluted with 3 volumes of distilled water and the mixture was adjusted to pH 6 with 1 N HCl. The solution was stirred overnight at 2℃ to precipitate ovomucin, which was removed by centrifugation at 3000×*g* for 5 min. The mucin-free supernatant was adjusted to pH 8.0 then applied, through two steps, to cation exchange chromatography on an S Hyper DF column (BioSepra, 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 OTF^B and OTF^C 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℃. Bacterial cells were suspended in trypticase soy broth (TSB; Nissui, Tokyo, Japan) to give 2×10^7 colony forming units (cfu)/m*l* (Ibrahim *et al*., 1996). Two-fold dilutions of ovotransferrin (1 mg/m*l* 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/m*l*). Controls were incubated with PBS instead of diluted ovotransferrin. The cultures in the microtiter plates were incubated at 37℃ 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 (ΔOD_{620}) in optical density, using the formula: $\Delta OD_{620} = OD_{620}$ at a given time – OD_{620} 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).

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^B, $OTF^{B/C}$ and OTF^C) were observed in two Satsumadori, Hua-Tung and Ju-Chi populations. The allele frequencies of OTF^C were 0.104, 0.238, 0.048 and 0.162 in the four populations, respectively. The OTF^B allele was fixed in two natives (Ehime-jidori and Tsin-Yi) and two commercial chickens. The OTF^A 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^B, $OTF^{B/C}$ and OTF^C. The *OTF* cDNA sequences for the phenotypes OTF^B, $OTF^{B/C}$ and OTF^C were deposited in the DDBJ database under accession numbers AB215094, AB 222603 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 AAT (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^B and OTF^C 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^B, $OTF^{B/C}$

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

Population	Number of samples	Phenotype OTF^B	Phenotype $OTF^{B/C}$	Phenotype OTF^C	Allele frequency *OTF^B*	Allele frequency *OTF^C*
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	*C 3354*	C 3412	*G 4734*	*C 5812*	G 7823	*G 8604*	G 10171
	(Ser52)	(Ile96)	(*Thr132*)	(Leu152)	(*Pro237*)	(*Ile364*)	(Asp500)	(*Gly510*)	(Ser686)
OTF^B	T (Ser)	A (Ile)	*C*	A (Ile)	*G*	*C/T*	G (Asp)	*G/A*	A (Asn)
$OTF^{B/C}$	T/G (Ser/Ala)	A/G (Ile/Val)	*C/G*	A (Ile)	*G/A*	*C/T*	G/A (Asp/Asn)	*G/A*	A (Asn)
OTF^C	G (Ala)	G (Val)	*C/G*	A (Ile)	*G/A*	*C/T*	A (Asn)	*G/A*	A (Asn)
Amino acid change	Ser>Ala	Ile>Val	*Thr*	Leu>Ile	*Pro*	*Ile*	Asp>Asn	*Gly*	Ser>Asn
Exon	Exon 2	Exon 3	*Exon 4*	Exon 4	*Exon 7*	*Exon 9*	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)		
		TT	*TG*	*GG*	*AA*	*AG*	*GG*	*GG*	*GA*	*AA*
OTF^{B}	11	8	3	0	11	0	0	11	0	0
$OTF^{B/C}$	17	0	15	2	7	10	0	0	17	0
OTF^{C}	1	0	0	1	0	0	1	0	0	1

Table 5. **Antibacterial effects of the purified OTF^{B} and OTF^{C} variants against *S. aureus* and *E. coli***

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

[a, b] Means (±SE) within a time and bacteria not sharing a common superscript differ ($P<0.05$). Values indicate the absorbance (A620nm) of the bacterial culture at the given time.
[1] Control was incubated with PBS instead of the purified ovotransferrin.

and OTF^{C} and agreed well with the phenotypes OTF^{B} (G/G, Asp500Asp), $OTF^{B/C}$ (G/A, Asp500Asn) and OTF^{C} (A/A, Asn500Asn) (Table 4).

Effect of the OTF Variants on the Antibacterial Activity

The OTF^{B} and OTF^{C} 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 (*S. aureus*) and Gram-negative (*E. coli*) 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/m*l*. 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 *S. aureus* and *E. coli*, indicating growth inhibition. The mean (±SE) bacterial growth of the OTF^{B} and OTF^{C} variants and control are shown in Table 5. Repeated measures ANOVA revealed that an interaction between variant and time was significant in both bacteria ($P<0.01$ in *S. aureus* and $P<0.05$ in *E. coli*). 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 ($P<0.05$ in *S. aureus* and $P<0.01$ in *E. coli*), suggesting that both purified ovotransferrin inhibited bacteria growth. However, no significant differences were found between the two variants in both bacteria ($P>0.05$).

Discussion

This study investigated the relationship between non-synonymous polymorphisms in the *OTF* gene and electrophoretic OTF variants and a difference among the variants for antibacterial activity. We focused on the difference between the variants OTF^{B} and OTF^{C}. This was because fresh eggs were necessary for antibacterial assay and no bird with OTF^{A} 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^{A} and OTF^{C} homozygous chickens because of low allelic frequencies. The genetic study for the OTF^{A} 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^{B}) to asparagine (AAT; allele OTF^{C}) 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^{C} 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 kringle 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 kringle 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 OTF^B variant at amino acid residue 500 to asparagine (Asn) in the OTF^C variant. The replacement of Asp500 with Asn500 reflects the difference in electrophoretic mobility between the OTF^B and OTF^C 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.

Acknowledgments

We express our thanks to Dr. Chih-Feng Chen (Department of Animal Science, National Chung Hsing University) for his preparation of blood and egg white samples of Taiwanese native chickens. We also express thank to Dr. J.J. Rodrigue for editing the English. This investigation was partly supposed by JSPS KAKENHI Grant Number 25450399 (to T.S.).

References

Baker CMA. Molecular genetics of avian proteins. IX. Interspecific and intraspecific variation of egg white proteins of the genus Gallus. Genetics, 58: 211−226. 1968.

Baker CMA, Croizier G, Stratil A and Manwell C. Identity and nomenclature of some protein polymorphisms of chicken eggs and sera. Advances in Genetics, 15: 147−174. 1970.

Baron F, Gautier M and Brule G. Rapid growth of Salmonella enteritidis in egg white reconstituted from industrial egg white powder. Journal of Food Protection, 62: 585−591. 1999.

Davis BJ. Disc electrophoresis. II. Method and application to human serum proteins. Annals of the New York Academy of Sciences, 121: 404−427. 1964.

Giansanti F, Rossi P, Massucci MT, Botti D, Antonini G, Valenti P and Seganti L. Antiviral activity of ovotransferrin discloses an evolutionary strategy for the defensive activities of lactoferrin. Biochemistry and Cell Biology, 80: 125−130. 2002.

Giansanti F, Massucci MT, Giardi MF, Nozza F, Pulsinelli E, Nicolini C, Botti D and Antonini G. Antiviral activity of ovotransferrin derived peptides. Biochemical and Biophysical Research Communications, 331: 69−73. 2005.

Ibrahim HR, Higashiguchi S, Koketsu M, Juneja LR, Kim M, Yamamoto T, Sugimoto Y and Aoki T. Partially unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism. Journal of Agricultural and Food Chemistry, 44: 3799−3806. 1996

Ibrahim HR, Iwamori E, Sugimoto Y and Aoki T. Identification of a distinct antibacterial domain within the N-lobe of Ovotransferrin. Biochimica et Biophysica Acta, 1401: 289−303. 1998.

Ibrahim HR, Sugimoto Y and Aoki T. Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through membrane damaging mechanism. Biochimica et Biophysica Acta, 1523: 196−205. 2000.

Ibrahim HR, Haraguchi T and Aoki T. Ovotransferrin is a redox-dependent autoprocessing protein incorporating four consensus self-cleaving motifs flanking the two kringles. Biochimica et Biophysica Acta, 1760: 347−355. 2006.

Ibrahim HR, Hoq MI and Aoki T. Ovotransferrin possesses SOD-like superoxide anion scavenging activity that is promoted by copper and manganese binding. International Journal of Biological Macromolecules, 41: 631−640. 2007.

Ibrahim HR and Kiyono T. Novel anticancer activity of the autocleaved ovotransferrin against human colon and breast cancer

cells. Journal of Agricultural and Food Chemistry, 57: 11383−11390. 2009.

Jacquinot PM, Leger D, Wieruszeski JM, Coddeville B, Montreuil J and Spik G. Change in glycosylation of chicken transferrin glycans biosynthesized during embryogenesis and primary culture of embryo hepatocytes. Glycobiology, 4: 617−624. 1994.

Keung WM and Azari P. Structure and function of ovotransferrin. II. Iron-transferring activity of iron-binding fragments of ovotransferrin with chicken embryo red cells. The Journal of Biological Chemistry, 257: 1184−1188. 1982.

Kinoshita K, Okamoto S, Shimogiri T, Kawabe K, Nishida T, Kakizawa R, Yamamoto Y and Maeda Y. Gene constitution of egg white proteins of native chicken in Asian countries. Asian Australasian Journal of Animal Sciences, 15: 157−165. 2002.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680−685. 1970.

Myint SL, Shimogiri T, Kawabe K, Hashiguchi T, Maeda Y and Okamoto S. Characteristics of seven Japanese native chicken breeds based on egg white protein polymorphisms. Asian-Australasian Journal of Animal Sciences, 23: 1137−1144. 2010.

Myint SL, Kinoshita K, Shimogiri T, Ibrahim HR, Tsusaki T, Tanoue T, Kawabe K, Maeda Y and Okamoto S. Effect of polymorphism in egg white lysozyme on muramidase and antibacterial activities as well as hatchability in the Japanese quail (*Coturnix japonica*). Journal of Animal Science, 90: 1747−1755. 2012.

Nei M and Chakraborty R. Genetic distance and electrophoretic identity of proteins between taxa. Journal of Molecular Evolution, 2: 323−328. 1973.

Oh TH, Markelonis GJ, Royal GM and Bregman BS. Immunocytochemical distribution of transferrin and its receptor in the developing chicken nervous system. Developmental Brain Research, 395: 207−220. 1986.

Oratore A, D'Andrea G, D'Alessandro AM, Moreton K and Williams J. Binding and iron delivering of monoferric ovotransferrins to chick-embryo red blood cells (CERBC). Biochemistry International, 22: 111−118. 1990.

Stevens L. Egg white proteins. Comparative Biochemistry and Physiology Part B, 100: 1−9. 1991.

Tanabe Y, Kano H, Kinoshita K, Taniwaki O and Okabayashi H. Gene Constitution of a Newly Found Population of Japanese Native Chickens in Southern Region of Ehime Prefecture, Shikoku, Japan. Japanese Poultry Science, 37: 101−107. 2000.

Thompson JD, Higgins DG and Gibson TJ. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673−4680. 1994.

Valenti P, Antonini G, Von HC, Visca P, Orsi N and Antonini E. Studies of the antimicrobial activity of ovotransferrin. International Journal of Tissue Reactions, 5: 97−105. 1983.

Williams J. A comparison of conalbumin and transferrin in the domestic fowl. Biochemical Journal, 83: 355−364. 1962.

Williams J. A comparison of glycopeptides from the ovotransferrin and serum transferrin of the hen. Biochemical Journal, 108: 57−67. 1968.