

Thermostabilization of ovalbumin by alkaline treatment: examination of the possible roles of D-serine residues

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Abstract: It was revealed from the crystal structure analysis of S-ovalbumin (S-OVA) formed by alkaline treatment that Ser164, Ser236, and Ser320 take the D-amino acid residue configuration (Yamasaki *et al.*, J Biol Chem 2003; 278:35524–35530). To address the implications of a D-configuration for these Ser residues in S-OVA formation, three mutant OVAs (S164A, S236A, and S320A) were generated to compare their thermostabilities before and after alkaline treatment. Following alkaline treatment, S236A showed a marked increase in melting temperature similar to the wild type (ΔT_m , +9°C) which corresponded to the formation of S-OVA, whereas the increment in T_m for both S164A and S320A was only 4.5°C. Furthermore, the T_m value of the double mutant S164/320A remained unchanged after alkaline treatment, supporting the relevance of Ser164 and Ser320 for thermostabilization of OVA. As Arg142 was predicted to interact with D-Ser164 upon S-OVA formation, it was substituted to Ala to generate R142A. The resulting increment in T_m of mutant R142A after alkaline treatment was 5.8°C. The double mutant R142/S320A was therefore prepared to eliminate the participation of Ser320 in thermostabilization, and its T_m value was compared before and after alkaline treatment. As expected, the increase in T_m for the double mutant was only 1.2°C. Taken together, the data suggest that D-configuration of Ser164 caused by alkaline treatment favors interaction with Arg142 through conformational changes of the side chain. These results strongly supported the participation of the configurational inversion of both Ser164 and Ser320 residues in the formation of S-OVA.

Keywords: ovalbumin; S-ovalbumin; D-amino acid configuration; alkaline treatment

General Statement

Ovalbumin is known to convert to thermostabilization form (S-form) under alkaline condition. However, the thermostabilization mechanism has been unclear. Recently, a crystallographic study for S-ovalbumin (S-OVA) formed by alkaline treatment revealed that Ser164, Ser236, and Ser320 take the D-amino acid residue configuration. However, there

is no experimental evidence that the chemical inversions contribute directly to the thermostability of S-OVA. Hence, we attempted to evaluate involvement of D-configurational inversion of the Ser residues upon the formation of S-OVA.

Introduction

Ovalbumin (OVA), a member of the serine protease inhibitor (serpin) superfamily, is converted into a thermostabilized form, S-OVA, during either the storage of unfertilized eggs or the development of fertilized eggs.^{1–3} Thermostabilization also occurs upon the *in vitro* incubation of isolated OVA under alkaline conditions.^{1,4} Although this interesting phenomenon has been extensively investigated by

Abbreviations: IPTG, isopropyl- β -D-thiogalactopyranoside; LB, Luria-Bertani; OVA, ovalbumin; S-OVA, stable ovalbumin; UV, ultraviolet; Serpin, serine protease inhibitor.

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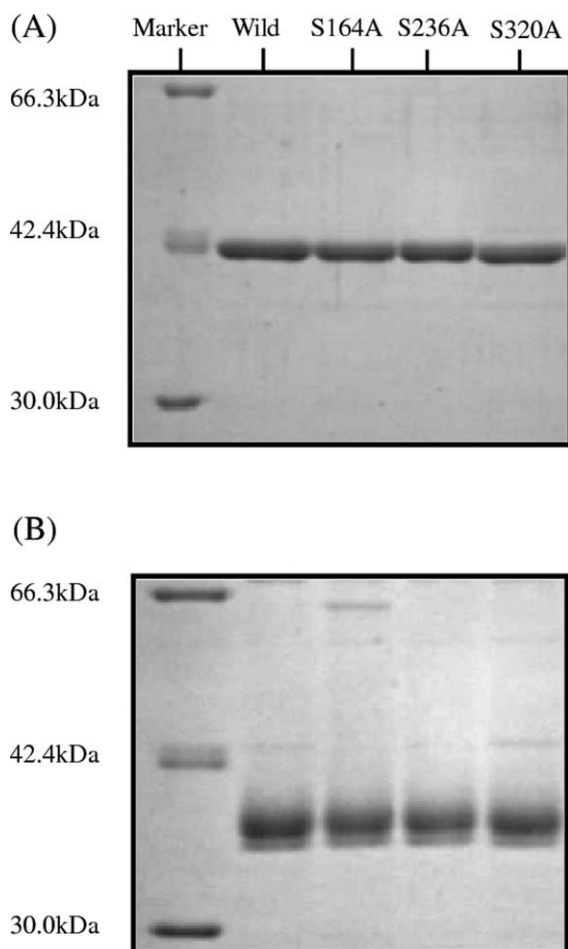


Figure 1. SDS-PAGE profiles of wild-type and three mutant OVAs. SDS-PAGE was performed under reducing (A) and nonreducing (B) conditions.

several researchers, the key elements involved in the stabilization of OVA have not been determined.

Recently, the crystal structure analysis of *S*-OVA at 1.9 Å resolution revealed that the Ser164, Ser236, and Ser320 residues take the *D*-amino acid residue configuration.⁵ In addition, conformational changes in the side chains of Phe99 and Met241 were identified as significant alterations in *S*-OVA.⁵ However, there is no experimental evidence that these chemical inversions are directly related to the structural stability of *S*-OVA. Thus, to elucidate the implications of the *D*-configuration of Ser residues for *S*-OVA formation and to analyze possible interactions in which *D*-Ser residues may be involved, we generated several recombinant OVAs in which a Ser residue was replaced with Ala. As Ser residues have the highest electron withdrawing capacity among nonglycine amino acids and are easily racemized under alkaline conditions, Ala substitutions were selected as they are resistant to racemization^{6,7} and hardly affect protein conformation.⁸

In this study, we attempted to determine if the thermostabilization mechanisms of *S*-OVA are directly related to *D*-configurational inversion of Ser

residues by examining the thermostabilities of recombinant OVAs before and after alkaline treatment.

Results

Structural properties of OVA

Arii *et al.*⁹ established an *Escherichia coli* expression system for OVA and reported that the conformation and disulfide structure of recombinant OVA were essentially identical to those of native OVA in egg white protein except for the lack of posttranslational modifications, such as N-terminal acetylation, phosphorylation, and glycosylation. In this study, recombinant OVAs (wild-type, S164A, S236A, and S320A) were isolated from the periplasmic fraction of *E. coli* cells using an osmotic shock procedure¹⁰ and then purified by ion-exchange chromatography. The homogeneity of these protein preparations was examined by SDS-PAGE (Fig. 1). The wild-type and mutant OVAs displayed a single band in SDS-PAGE gels in the presence of 2-mercaptoethanol, demonstrating their homogeneity and success of the purification [Fig. 1(A)]. As observed in Figure 1(B), each of the recombinant proteins was observed as a faster-migrating band in SDS-PAGE gels in the absence of reducing reagent, suggesting the presence of intramolecular disulfide bonds.

As the far-ultraviolet (UV) CD spectrum is reliable for evaluating the secondary structure of proteins, the CD spectra for the wild-type and mutant OVAs were determined (Fig. 2). The CD profile of the wild-type OVA protein expressed and purified from *E. coli* was indistinguishable from native OVA isolated from egg whites (data not shown). As shown in Figure 2(A), the CD profiles of the mutant proteins S164A, S236A, and S320A were similar and were nearly identical to those of wild-type OVA.

OVA has three tryptophan residues, Trp148 on helix F, Trp184, which is the nearest neighbor residue of the C-terminus of strand 3A, and Trp267 on helix H.¹¹ To evaluate the conformation of OVA, the environment surrounding these tryptophan residues was examined by monitoring the intrinsic fluorescence of the wild-type and recombinant proteins. The wild-type OVA displayed almost the identical fluorescence emission spectrum as egg white OVA, with an emission maximum at 342 nm (data not shown). As shown in Figure 3(A), the fluorescence emission spectrum of each mutant protein (S164A, S236A, and S320A) was fairly similar to that of wild-type OVA which suggests that each mutant OVA assumes a native-like conformation.

Thermostability

As shown in Figure 4, thermal unfolding curves for the wild-type and mutant OVA proteins were generated by monitoring the change in CD ellipticity at

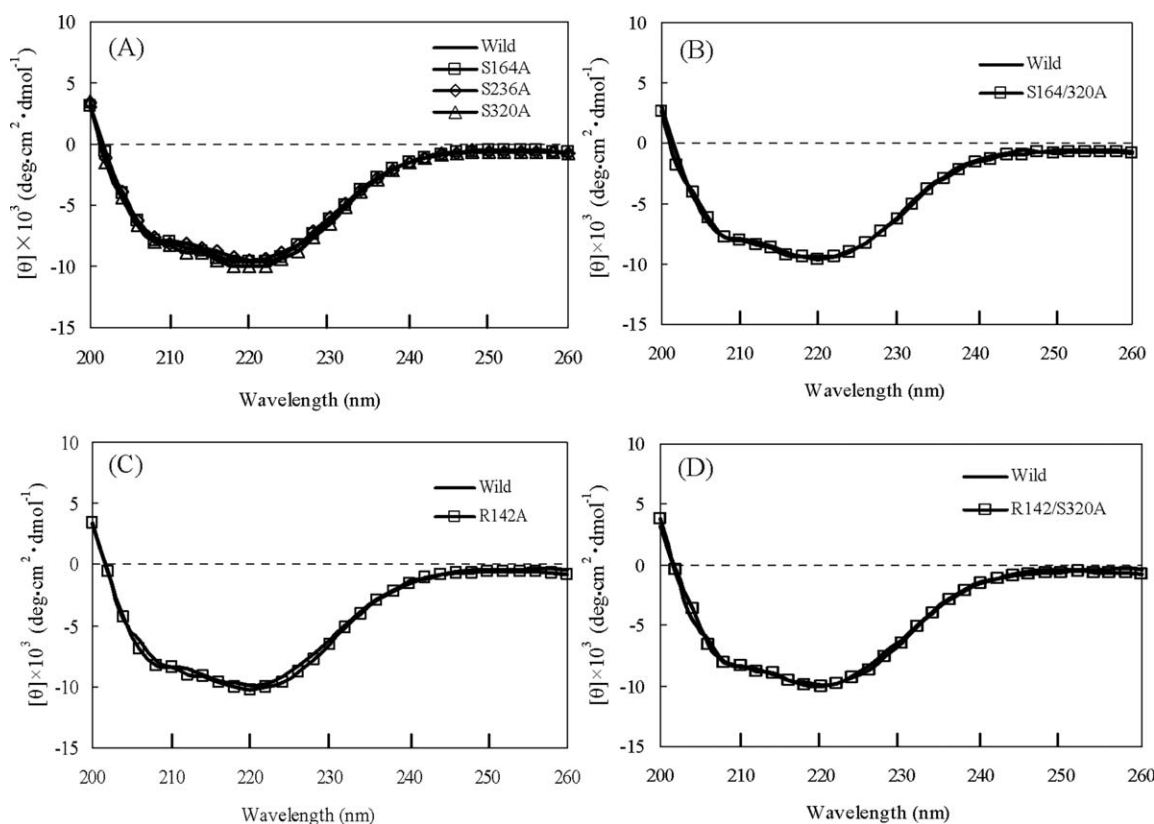


Figure 2. Far-UV CD spectra of wild-type and various mutant OVAs. CD spectra were recorded in 10 mM potassium phosphate buffer, pH 7.0. (A) Wild-type, S164A, S236A and S320A. (B) Wild-type and S164/320A. (C) Wild-type and R142A. (D) Wild-type and R142/S320A.

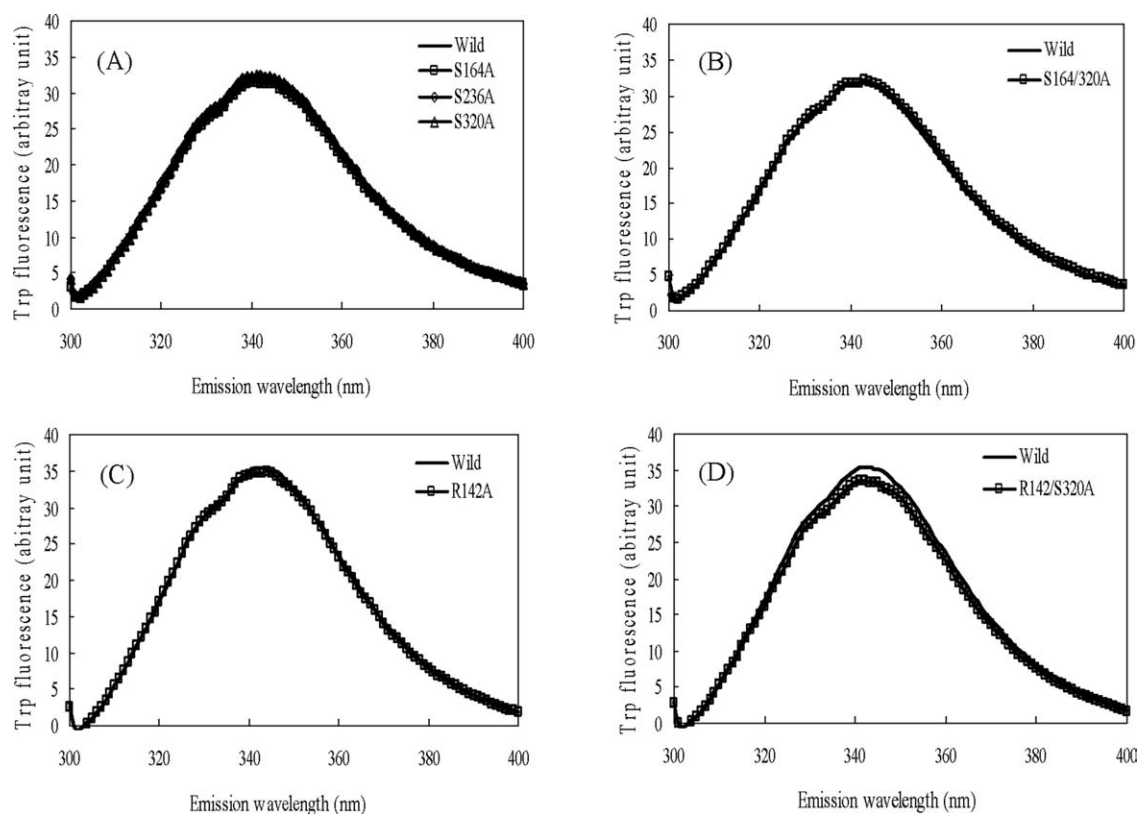


Figure 3. Tryptophan fluorescence emission spectra of wild-type and various mutant OVAs. Fluorescence spectra were recorded in 10 mM potassium phosphate buffer, pH 7.0, at an excitation wavelength of 295 nm. (A) Wild-type, S164, S236 and S320. (B) Wild-type and S164/320A. (C) Wild-type and R142A. (D) Wild-type and R142/S320A.

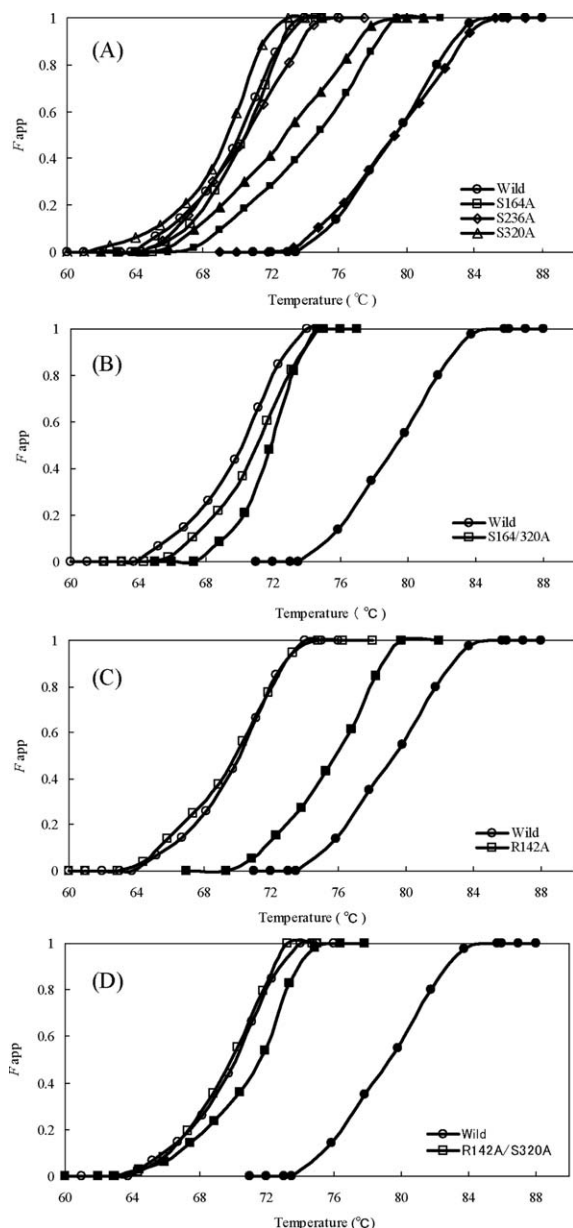


Figure 4. Temperature-dependent unfolding of wild-type and various mutant OVAs. F_{app} was deduced from the change in ellipticity at 222 nm. The temperature was increased from 25 to 80°C at a rate of 1°C/min for the heat-denaturation process. Open and closed symbols represent the intact and alkaline-treated proteins, respectively. (A) Wild-type, S164A, S236A and S320A. (B) Wild-type and S164/320A. (C) Wild-type and R142A. (D) Wild-type and R142/S320A.

222 nm observed at different temperatures. It was confirmed that the unfolding could be approximated by the mechanism for a two-state transition between the folded and unfolded states. Thus, the mid-point temperature (T_m) of thermal unfolding was deduced from each unfolding curve. Aii *et al.*⁹ demonstrated that recombinant OVA expressed in *E. coli* displayed a T_m value of 72.5°C as evaluated by DSC analysis, and was transformed into heat-stable S-OVA ($\Delta T_m = 8^\circ\text{C}$) upon alkaline treatment. The wild-type OVA

prepared from the *E. coli* periplasmic fraction in this study had a T_m value of 69.8°C as determined from the thermal unfolding curve [Fig. 4(A)], which was 2.7°C lower than the value reported by Aii *et al.*⁹

To confirm the formation of the S-form of wild-type OVA, the purified protein was subjected to alkaline treatment. The alkaline-treated wild-type OVA showed a dramatically increased T_m value of 78.9°C [Fig. 4(A), Table I]. The obtained T_m values for the intact and alkaline-treated recombinant OVAs are summarized in Table I. The T_m values for the intact OVA mutants (70.8°C for S164A, 70.4°C for S236A, and 69.3°C for S320A) were fairly similar to that of intact wild-type (69.8°C). The data imply that mutation of the Ser residue to Ala does not affect the conformational stability of OVA. In contrast, upon alkaline treatment, the T_m for S236A increased by 8.9°C, which was similar to the case of wild-type OVA. However, the increment in T_m for S164A and S320A was only 4.8°C and 4.5°C, respectively, corresponding to approximately half of the increase observed for wild-type OVA. This suggests that the D-configuration of Ser164 and Ser320 contribute to the thermostabilization of OVA, but that of Ser236 does not. To eliminate the contribution of the Ser164 and Ser320 residues to OVA thermostabilization, an Ala double mutant (S164/320A) was generated and characterized. The double mutant displayed the identical far-UV CD profile, intrinsic fluorescence, and thermostability as wild-type OVA [Figs. 2(B), 3(B), and 4(B)], indicating that the mutation did not induce significant changes in the protein conformation. As expected, alkaline treatment did not affect the T_m value of the double mutant. Taken together, these data demonstrate that D-configurational inversion of Ser residues at positions 164 and 320 participates in the stabilization of S-OVA.

A possible mechanism for thermostabilization

The observed increase in T_m for alkaline-treated OVA was thought to be equivalent to the regeneration of at least one noncovalent bond. The partner residue for Ser164 was therefore explored using molecular modeling. Arg142 was targeted as the most probable partner for D-Ser164 as it is a bulky residue that exists in the vicinity of Ser164 and is located in helix F, which is important for the stability and inhibitory activity of serpins.^{12,13} The mutant R142A was therefore prepared and its structural properties were compared to wild-type OVA. As shown in Figures 2(C), 3(C), and 4(C), the overall structure and T_m of R142A were very similar to those of wild-type OVA. After alkaline treatment, the increase in T_m ($\Delta 5.8^\circ\text{C}$) for R142A was considerably lower than that observed for wild-type OVA [Fig. 4(C), Table I]. The data supported our expectation that the conformational changes in the side chain of Ser164 induce the increased interactions

Table I. Thermal Denaturation Temperature (T_m) of Wild and Various Mutant OVAs Before and After Alkaline Treatment

	Intact (°C)	Alkaline treated (°C)	ΔT_m (°C)
Wild	69.8	78.9	+ 9.1
S164A	70.8	75.6	+ 4.8
S236A	70.4	79.3	+ 8.9
S320A	69.3	73.8	+ 4.5
S164/S320A	71.3	71.8	+ 0.5
R142A	69.8	75.6	+ 5.8
R142/S320A	70.0	71.2	+ 1.2

with Arg142. To eliminate the contribution of Ser320 to thermostabilization, both Arg142 and Ser320 were substituted with Ala (R142A/S320A) and the conformation of the double mutant protein was then compared to that of wild type. As can be seen in Figures 2(D), 3(D), and 4(D), R142A/S320A displayed a similar conformation and T_m (70.0°C) to wild-type OVA. However, following alkaline treatment, the observed increment in T_m for R142A/S320A was only 1.2°C (Table I). This data strongly suggest that D-configurational inversion of Ser164 upon alkaline treatment participates in the thermostabilization of OVA through an increased interaction with Arg142.

Discussion

As OVA, a member of the serpin superfamily, was first reported to be converted into a thermostabilized form, S-OVA, many investigators have extensively studied the mechanism of S-OVA formation. Recently, a crystallographic study of S-OVA formed by alkaline treatment revealed that Ser164, Ser236, and Ser320 take the D-amino acid residue configuration.⁵ Further, Hirose and colleagues showed that denatured S-OVA refolded correctly into the original nondenatured form with restored thermostability and demonstrated that configurational inversion plays a central role in the thermostabilization of S-OVA.¹⁴ However, there was no experimental evidence that chemical inversions contributed directly to the thermostability of S-OVA. Hence, to address the implications of D-configurational inversion of the Ser residues upon formation of S-OVA, we prepared recombinant OVAs with Ala substitutions at each Ser residue to eliminate the effects of chemical inversion, and determined their thermostabilities after alkaline treatment.

Considering the analyses of CD and intrinsic tryptophan fluorescence as shown in Figures 2 and 3, it appears that the substitution of the selected Ser residues with Ala does not affect the gross conformation of OVA. Moreover, It was found that wild-type OVA prepared in this study can be converted into S-form ($\Delta T_m = 9.1^\circ\text{C}$) with a corresponding high thermostability upon alkaline treatment.

As the increases in T_m were only approximately half of the wild-type value after alkaline treatment, we propose that D-configuration of Ser164 and

Ser320 is structurally important for the thermostabilization of OVA. In a previous study, it was reported that during the transformation from OVA to S-OVA a thermodynamically distinct intermediate (I-OVA) was involved.¹⁵ The thermostability of I-OVA was shown to be 4°C higher than that of native OVA, corresponding to half of the ΔT_m for S-OVA.¹⁴ Thus, it seems reasonable to speculate that either D-configurational inversion conformer of Ser164 or Ser320 may correspond to an I-OVA generated by a stepwise process during the formation of S-OVA.

To certificate a possible partner residue for D-Ser164 in thermostabilized OVA, we prepared a R142A mutant in which the bulky Arg142 residue on helix F was replaced by the less bulky Ala. As expected, after alkaline treatment of the mutant R142A, an increment in T_m of 5.8°C was observed, corresponding to approximately half of the value of alkaline-treated wild-type OVA. Hence, we assumed that D-configurational inversion of the Ser164 side chain may induce increased accessibility to Arg142 adding to the strength of helix F packing. To confirm this postulation, the possibility of a hydrogen bond between O (D-Ser164) and N (Arg142) in S-OVA was analyzed by molecular modeling. From the crystallographic data, the distance between these atoms was estimated to be 6.86 Å which is too great a distance to form a hydrogen bond. Moreover, with regard to the configurational inversions of the three Ser residues, Yamasaki *et al.*⁵ demonstrated from crystal structure analyses that the Ser164, Ser236, and Ser320 residues reside on the protein surface in both native OVA and S-OVA and do not have any direct interactions with surrounding residues. Thus, in an attempt to resolve this discrepancy, we focused on the water molecules surrounding residue Ser164. Refined crystallographic data at 1.95 Å and 1.9 Å resolution are reported for OVA (PDBID: 1OVA) and for S-OVA (PDBID: 1OH) respectively, providing the positions of water molecules in their proteins, as shown in Figure 5.^{5,16} This approach allowed the identification of a water molecule near the O atom of Ser164 [in Fig. 5(B,C), crystallographic water molecules are represented by dotted spheres]. From the positioning of a water molecule in the vicinity of Ser164, it can be supposed that the OH-group of D-Ser164 is bound to either the side chain or backbone of Arg142 via water-mediated hydrogen bonds which contribute to the structural stability of S-OVA. However, as this was not conclusively demonstrated in this study, further experiments are needed to verify this speculation. Further, as another significant alteration observed in the crystal structure analysis of S-OVA, changes in the side-chain conformation of Phe99 on strand 2A and Met241 on strand 3B also occur.⁵ As Ser164 and Ser320 residues exist in the vicinity of Arg142 on helix F and Phe99 on strand 2A, respectively, the D-configurational inversions of

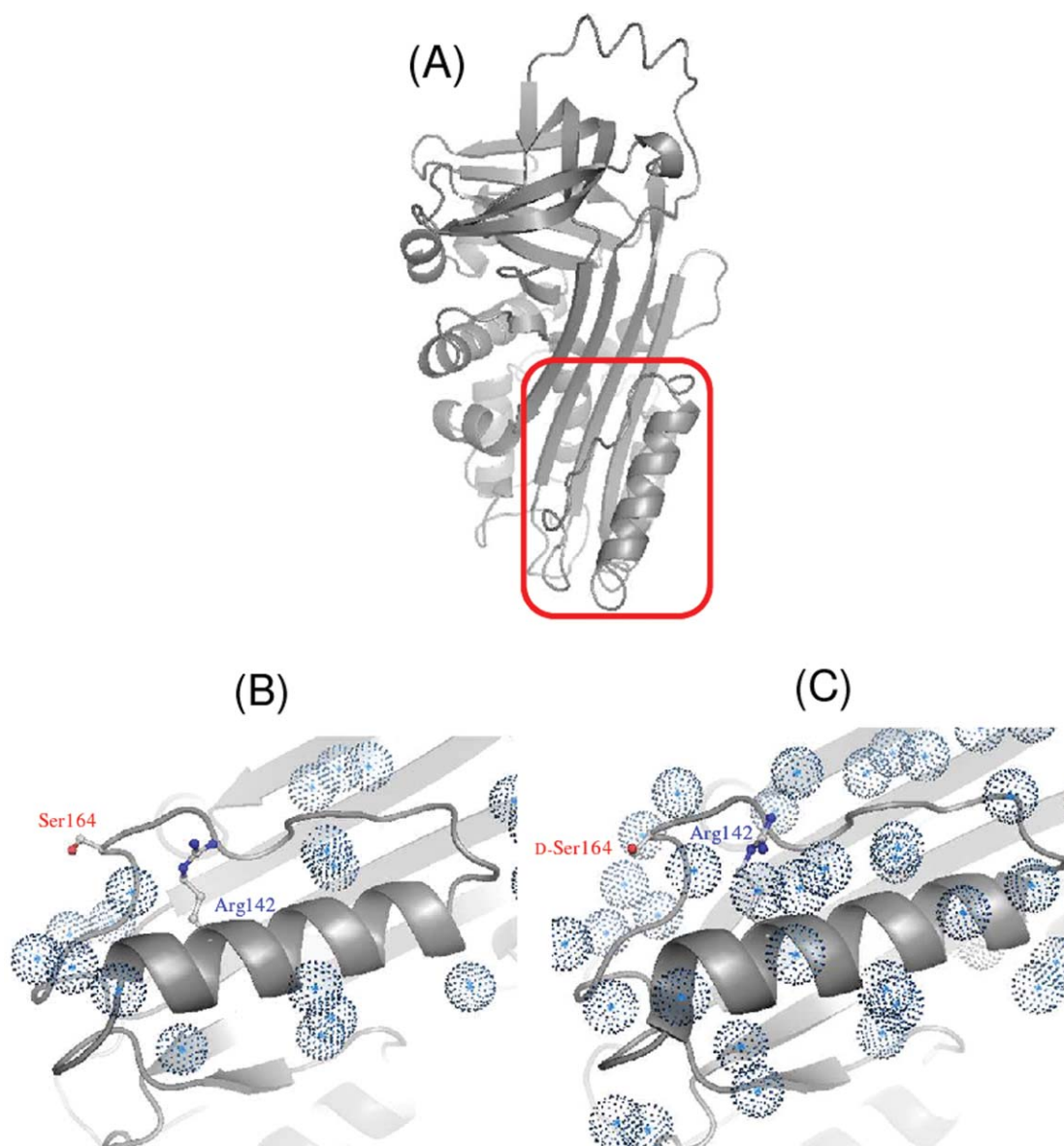


Figure 5. Predicted structures of OVA and S-OVA. The predicted structures are based on the crystallographic data of OVA (A, B) (Protein Data Bank code 1OVA)¹⁶ and S-OVA (C) (Protein Data Bank code 1UHG)⁵ and were drawn using PyMOL.¹⁷ The dotted spheres represent water molecules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Ser164 and Ser320 may induce concomitant changes in the side-chain conformation of both Phe99 and Met241.

In conclusion, our study strongly supports the results of crystal structure analyses for S-OVA that Ser164 and Ser320 take the D-amino acid configuration. It was also certificated that the configurational inversions of the Ser164 and Ser320 residues play a central role for the formation of the thermostabilized OVA.

Materials and Methods

Materials

Restriction enzymes, ligase, and polymerase were purchased from Takara Shuzo (Kyoto, Japan). Oligonucleotide primers were provided by Sigma-Aldrich

Japan K. K. Genosys Division. A DNA sequencing kit was obtained from PE Biosystems (Foster City, CA). Carbenicillin, isopropyl- β -D-thiogalactopyranoside (IPTG), and phenylmethylsulfonyl fluoride were obtained from Nacalai Tesque (Kyoto, Japan). Egg white OVA was prepared from fresh egg whites by crystallization in an ammonium sulfate solution.¹⁸ All other chemicals used in the experiments were of analytical grade.

RNA extraction and cloning of hen OVA gene

Hen oviducts were used for the extraction of total RNA according to the protocol of the Qiagen RNeasy Total RNA purification kit. The extracted RNA was subsequently treated with RNase-free DNase

according to the manufacturer's instructions (Promega) and used for RT-PCR using a Qiagen One-Step RT-PCR kit. Primers for amplifying the hen OVA gene were 5'-ATGGGCTCCATCGGCGCAGC-3' (sense) and 5'-TTAAGGGGAAACACATCTG-3' (anti-sense). RT-PCR products were amplified with Taq DNA polymerase and further purified using a PCR product purification kit (Boehringer). RT-PCR fragments were subjected to a mung bean nuclease treatment according to the manufacturer's instructions (Promega). After ligation into plasmid PT7Blue, PCR fragments were sequenced using an Applied Bio System DNA sequencer (ABI PRISM R-310). The sequence of the OVA gene was identical to the one already submitted to the GenBank nucleotide sequence data base under accession number V00383.

Strains and plasmids

Escherichia coli strains XL1-Blue and BL21 were used as host cells for cloning experiments and OVA expression, respectively. pT7 Blue T vector was used for the subcloning of OVA cDNA and was obtained from Novagen Merck (Darmstadt, Germany). The expression plasmid pET-22b(+) containing the IPTG-inducible T7 promoter and ampicillin resistance gene was purchased from Invitrogen. The signal sequence pelB reader in pET-22b(+) was removed because OVA contains a native secretion signal sequence.¹⁹

Vector construction and protein expression

Each mutant protein, in which the following Ser residues in OVA were replaced by Ala, was constructed by PCR using the synthetic oligonucleotide primers 5'-CAGCCTCCGTGGATTCTCAA-3' (sense) and 5'-AGGCTGGCTGAAGGACATTT-3' (anti-sense) for S164A, 5'-CCGCTGGGACAATGAAGCATG-3' (sense) and 5'-CCAGCGGCAAATGGAAGCTC-3' (anti-sense) for S236A, and 5'-AGGCCCTGAAGATATCTCAAGC-3' (sense) and 5'-AGGGCCTCTGCTGAGGAGAT-3' (anti-sense) for S320A (underlines indicate a mutation site). In addition, the R142A mutant was constructed using oligomer primers 5'-GCCGCAGAGCTCATCAATT-3' (sense) and 5'-CTCTGCGGCTTGATCTGC-3' (anti-sense) for the mutation of R (underlined nucleotides). Point mutations were performed using Ex Taq polymerase and pT7 Blue containing OVA cDNA as a template. After the template was digested with *DpnI*, the plasmid carrying mutated OVA cDNA was transformed into *E. coli* XL1-Blue competent cells according to the method of Hanahan.²⁰ Transformants were selected using Luria-Bertani (LB) medium plates containing 50 µg/mL carbenicillin. Subsequently, the ovalbumin cDNA was excised from pT7 Blue by digestion with *NdeI* and *SalI*, and then ligated to pET-22b(+) with T4 DNA ligase. The plasmid carrying OVA cDNA was trans-

formed into *E. coli* XL1-Blue competent cells and transformants were again selected using LB plates containing carbenicillin. A few potential transformants for each mutant were selected and incubated in test tubes to assess their expression levels. The quantity of expressed protein was examined by SDS-PAGE, and the strain with the highest level of expression was selected for each mutant and used for subsequent mass culture. *E. coli* cells carrying pET22b(+)/OVA were inoculated into 5 mL of LB medium supplemented with 50 µg/mL carbenicillin and incubated overnight at 37°C while shaking. The culture was then transferred into a shake flask containing 500 mL of LB medium and incubated under identical conditions for 3 h. Fifty microliters of 1 M IPTG was then added into the LB medium to induce the expression of OVA and further incubated for 3 h. The resulting culture was then subjected to protein purification.

Protein purification

Following the overexpression of OVA protein in the 500 mL broth cultures, the cultures were centrifuged to harvest the cells. The cells were then subjected to osmotic shock treatment described by Neu and Heppe¹⁰ for the fractionation of periplasm. The obtained periplasmic fraction was used for the purification of OVA by DEAE-Sepharose FF column chromatography with a linear gradient of 0 to 0.2 M NaCl in 10 mM phosphate buffer (pH 7.0). To visualize proteins, SDS-PAGE was performed using 10% polyacrylamide gels under both reducing and nonreducing conditions by the standard method described by Laemmli²¹ and stained with Coomassie Brilliant Blue R-250.

Alkaline treatment for S-OVA formation

To prepare S-OVA, an alkaline treatment was conducted essentially as described by Smith and Back,¹ but with the modifications reported by Yamamoto *et al.*²² Briefly, protein samples were suspended at 1.0 mg/mL in 0.1 M glycine-NaOH buffer containing 0.02% sodium azide at pH 10 and incubated at 50°C for 24 h. The alkaline-treated sample was then passed through a Sephadex column (NAP-10, Amersham Biosciences) equilibrated with 10 mM phosphate buffer, pH 7.0, and subjected to analyses by CD and intrinsic tryptophan fluorescence.

Measurements of CD

CD spectra were recorded with a spectropolarimeter (model J-600; Jasco, Tokyo, Japan) equipped with an interface and a thermostatically controlled cell holder. Unfolding transition curves were obtained by measuring the ellipticity at 222 nm at a protein concentration of 0.1 mg/mL in a 1-mm cell. The temperature was increased from 25°C to 90°C at a rate of 1.0°C/min for the heat-denaturation process. The

change in temperature was monitored by a thermostat sensor in the cell holder. The measured ellipticities were corrected manually for the monitored temperatures. Assuming that the unfolding equilibrium followed a two-state mechanism, the mid-point temperature (T_m) of unfolding was determined from the obtained unfolding curves.

Far-UV CD spectra were recorded at a protein concentration of 0.25 mg/mL with a 1-mm cell at wavelengths ranging from 260 to 200 nm at 25°C. The data were collected in triplicate and expressed as the mean residue ellipticity.

Measurement of fluorescence

Tryptophan fluorescence spectra were recorded with a fluorescence spectrophotometer (model FP-6300; Jasco) at 25°C using samples at a concentration of 0.25 mg/mL in 10 mM phosphate buffer (pH 7.0). The excitation and emission wavelengths were 295 and 300–400 nm, respectively.

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