Studies on the Cellular Localization and Thermostability of Ovalbumin Produced in *Escherichia coli*

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CONTENTS

ABBREVIATIONS

CD	circular dichroism
DSC	differential scanning calorimetry
DTT	dithiothreitol
E. coli	*Escherichia coli*
ER	endoplasmic reticulum
IAA	monoiodoacetic acid
IAEDANS	N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylene di-amine
IAM	iodoacetamide; LB, Luria-Bertani (medium)
LB	Luria-Bertani (medium)
MES	2-(N-morpholino) ethanesulfonic acid
Ova	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
PPE	porcine pancreatic elastase
SCMC	S-carboxymethyl cysteine
Serpin	serine proteinase inhibitor
TFA	trifluoroacetic acid

INTRODUCTION

Ovalbumin, which is the major component of egg white proteins, shows unique characteristics in the secretion and thermostability.

First, ovalbumin is secreted without any cleavages of the signal sequence for the secretion in hen oviducts (Palmiter et al., 1978). Secretory protein adds a signal sequence for secretion to N-terminus of the mature protein (Hartl & Wiedmann, 1993). The protein is usually transported into the endoplasmic reticulum (ER) lumen by the cleavage of the signal sequence (Schatz & Dobberstein, 1996). On the contrary, ovalbumin has an internal signal sequence, which is localized within a hydrophobic region comprising the first 22 to 41 amino terminal residues in the mature protein (Tabe et al., 1984). The protein is secreted without any cleavage of the signal region.

Second, ovalbumin transformed into a more heat stable form, S-ovalbumin, accompanying a rise in the pH value of egg white during stored shell eggs (Smith & Back, 1965) and on the process of embryogeny (Sugimoto et al., 1999). This transformation is also caused *in vitro* by an alkaline treatment. Although the transition mechanism has been investigated by various studies but not made it clear. It is suggested that the post-translational modifications known in egg white protein have any effects on the mechanism and the conformatio-nal change of ovalbumin takes part in the mechanism (Huntington et al., 1995a).

Last, ovalbumin is a member of serine proteinase inhibitor (serpin) superfamily but does not show any inhibitory activities (Hunt & Dayhoff, 1980). The expression of serpin activity accompanies a large scale conformational change that is insertion of the reactive loop into β-sheet by the cleavage at the reactive center and transformed into a more stable form (Stein et al, 1989).

Three-dimensional X-ray crystallographic structure information of ovalbumin shows to include specific structures in serpin, which has three β-sheets and nine α-helices (Stein et al., 1991). However, ovalbumin does not undergo the dynamic conformational change and thermostabilized upon the cleavage of the serpin P1-P1' site.

In this study, the author examined the secretion into periplasm and the thermostabilization under the alkaline condition with an expression system of recombinant ovalbumin in *Escherichia coli* (*E. coli.*) Furthermore, the author made an ovalbumin variant by a site-directed mutagenesis and investigated changes in thermostability, which is used as an indicator of the conformational change like serpin.

In chapter 1, the author made it clear that the recombinant protein is secreted into periplasmic space of *E. coli* cells without any cleavage of N-terminal region. The secreted protein did not contain the disulfide bond. Further, a mutant that is deleted N-terminal region was not secreted into periplasmic space *in E. coli.*

In chapter 2, the recombinant ovalbumin was purified from the cytoplasm of *E. coli*, and its structural characteristics are compared with those of egg white ovalbumin. The conformation and disulfide structure of the recombinant protein is essentially the same as those of egg white protein. However, none of the post-translational modifications known in egg white ovalbumin, including N-terminal acetylation, phosphorylation, and glycosylation, were detected in the recombinant protein. Furthermore, the recombinant protein was transformed into a more thermostable form by an alkaline treatment.

In chapter 3, the author is prepared an ovalbumin variant that was changed an amino acid residue on the reactive center loop by site-directed mutagenesis and indicated the possibility that the dynamic conformational change is occurred in the variant. Moreover, the author established the

method in kinetics of loop insertion by proteolytic cleavage analysis and estimated rate constants for loop insertion and proteolytic cleavages. Using the kinetic method, the loop-insertion mechanism in the ovalbumin variant was investigated.

CHAPTER 1.

Cellular localization of recombinant ovalbumin in *Escherichia coli.*

Secretory proteins in bacteria were passed across the plasma membrane (Wickner et al., 1991). The secretory process is evolutionarily related to the transport of proteins across the membrane of endoplasmic reticulum in eukaryotic cell (Schatz & Dobberstein, 1996). In both systems, proteins destined for secretion are usually synthesized as precursor, which contains N-terminal signal sequence that is functionally interchangeable between prokaryotic and eukaryotic cells (Hartl & Wiedmann, 1993). These signal peptides, which are later removed from mature protein by signal peptidase, contain a hydrophobic region of 16-26 amino acids (Wickner et al., 1991). In addition, many secretory proteins contain disulfide bonds, which are important for their folding, stability, and appearance of function, in prokaryotic and eukaryotic cells (Ellis & Hartl, 1999). The disulfide formation is going on the secretory process. In *E. coli*, the formation of disulfide bond is carried out by a specific Dsb-system in the periplasmic space (Bardwell et al., 1993). While in the eukaryotic cells, the disulfide formation is facilitated by an enzyme, protein disulfide isomerase, in endoplasmic reticulum (Zapun et al., 1999).

Ovalbumin is secreted by the tubular gland cells without any cleavage of a signal sequence at the N-terminus (Palmiter et al., 1978) and has four cystein residues (Cys11, Cys30, Cys367, and Cys382) and a cystine (Cys73-Cys120) (Forthergill & Forthergill, 1970). By secretion analyses using a *Xenopus* oocyte expression system, ovalbumin has been shown to possess an internal signal that is localized within a hydrophobic region comprising the first 22 to 41 amino terminal residues (Tabe et al., 1984). In previous reports, ovalbumin synthesized in *E. coli* has been shown to be secreted into periplasmic space (Fraser &Bruce, 1978; Mercereau-Puijalon et al., 1978; Baty et al.,

1981). The expression plasmid has, however, included 19 residues of N-terminal extension that are derived from β-galactosidase (Fraser &Bruce, 1978) or the loss of four residues of the original N-terminus of ovalbumin as well as the extension of nine residues from β-galactosidase sequence (Mercereau-Puijalon et al., 1978; Baty et al., 1981). In these previous reports, neither conformational property nor disulfide formation has been examined.

In many bacterial expression systems, recombinant eukaryotic proteins, which contain the disulfide bond, was secreted into periplasm with prokaryotic signal sequence and facilitated the formation of disulfide bond using a specific Dsb system (Hockney, 1994). The full length of ovalbumin has previously been expressed using the *E. coli* expression system space (Takahashi et al., 1995) without any prokaryotic signal sequence for the secretion into periplasmic. The recombinant protein purified from total cell fraction indicated the same secondary and ternary structure as egg white ovalbumin (Takahashi et al., 1995). In this chapter, the author demonstrated that recombinant ovalbumin, which contains no additional peptide and prokaryotic signal, was secreted into the periplasmic space without any cleavage of N-terminal region, and the disulfide bond could not form using any disulfide-forming systems in the *E. coli* cell.

Materials and Methods

Materials.

Ovalbumin was purified from fresh egg white by crystallization three times (Söresen & Höyrup, 1915). N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylene di-amine (IAEDANS) was purchased from Aldrich Co. Other chemicals, including dithiothreitol (DTT), urea, iodoacetamide (IAM) and monoiodoacetic acid (IAA), were of guaranteed grade from Nacalai Tesque. Trypsin (type XI) and chymotrypsin (type II) were purchased from Sigma Co. Achromobacter protease I

(EC 3.4.21.50) was obtained from Wako Pure Chemical Industries. Oligonucleotides were purchased from Amersham Pharmacia Biotech.

Plasmids and strains.

pET3d vector was purchased from Novagen. The plasmid inserted ova gene, pCR1ov2.1. (Humphries et al., 1997), was gifted from Dr. P. Chambon (Laboratorire de Genstique Moleculair des Eucaryotes du CNRS, Strasbourg, France). Expression vector, pET/Ova has been constructed by Takahashi et al. (1995). pET/Ova(Δ1-39) was inserted a fragment containing an Ova gene, which was excised from pCR1ov2.1. with *Nco*I and *BamH*I digestion and was not encoded the N-terminal amino acid residues from Gly1 to Ala39 into pET3d treated with the same digestion. *E. coli* strain BL21(DE3) was used for expression of ovalbumin gene.

Cellular fractionation of recombinant ovalbumin.

To investigate the cellular distribution of recombinant protein, *E. coli* harboring pET/Ova was inoculated in 16 ml of LB medium supplemented by 50 μg/ml of ampicillin and incubated at 37 °C with agitation for 16 h. The periplasmic fraction was obtained after osmotic shock treatments of the cells (Neu & Heppel, 1965): *E. coli* cells collected by centrifugation (8,000 rpm, 5 min) from the 8 ml of culture were rinsed with 1 ml of buffer A (50 mM Na-phosphate buffer (pH 6.0) containing 20 % sucrose and 1 mM Na-EDTA), suspended and incubated in buffer A at 25°C for 20 min, and centrifuged. The precipitated cells were resuspended in 1 ml of cold distilled water, incubated at 0°C for 20 min, and centrifuged. The supernatant was stored as the periplasmic fraction. The precipitated cells were suspended in 1 ml of 50 mM Na-phosphate buffer (pH 6.0) containing 1.5 mM phenylmethylsulfonyl fluoride and 1 mM Na-EDTA, disrupted with a sonicator

(SONIFIER 250, BRANSON) for 1 min 20 times at 0°C, and then centrifuged. The clear supernatant was used as the cytoplasmic fraction. The proteins in 10 μl of the periplasmic and cytoplasmic fractions were used for an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to investigate the localization of recombinant protein and ovalbumin bands were made visible by a western analysis. To analyze the intrachain disulfide formation in the periplasm, 0.5 ml of the supernatant after the distilled cold water treatment was added 15.9 μl of 1 M Tris-HCl (pH 8.0) and 13.1 μl of 2M IAA, and incubated for 15 min at 25 °C. For marker enzyme assays (β-galactosidase for cytoplasm (Craven et al., 1965) and alkaline phosphatase for periplasm (Garen & Levinthal, 1960)), 0.125 ml of the fractions were employed.

SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE was carried out with 10% polyacrylamide gel at a constant current of 12.5 mA for 2.5 h according to the standard method by Laemmli (1970). Proteins were made visible by a western blotting analysis, using the ovalbumin specific antiserum as described previously (Takahashi et al., 1992) and quantified using a flatbed scanner (GT-9000, Epson) and the software NIH image (version 1.60).

Purification of recombinant ovalbumin.

For the purification of recombinant protein from periplasmic space, the 2 liters culture was used for the osmotic shock treatments. The periplasmic fraction was obtained by the same way with 100 ml of cold distilled water following to incubate in 100 ml of buffer A. The periplasmic proteins were concentrated by precipitation in ammonium sulfate solution (90 % saturation) and subsequent solubilization in 10 mM Na-phosphate buffer, pH 6.0. The protein samples were

dialyzed against the same buffer, applied to an anion exchange column (HiLoad 26/10 Q-Sepharose HP, Amersham Pharmacia Biotech.) and eluted by a linear 0.01 to 0.15 M gradient of Na-phosphate buffer, pH 6.0 (Takahashi et al, 1995). Ovalbumin peaks as detected by the western blotting analysis using an ovalbumin antibody were collected. Maltose binding protein that was present in the periplasmic ovalbumin sample was removed by passage through an amylose-resin column (New England BioLabs). The purified proteins were concentrated by precipitation in ammonium sulfate solution in the same way. The protein concentration was estimated for recombinant and egg white ovalbumin from absorption at 280 nm using $E^{1\%}_{1\,cm} = 7.12$ (Glazer et al., 1963).

Conformational analyses.

The N-terminal amino acid sequence was determined with a protein sequenator (Procise 492, PE Applied Biosystems). The identification for disulfide-forming half-cystines was performed by protein alkylation with a fluorescent reagent, N-iodoacetyl-N'-(5-sulfo-1-naphthyl) ethylene-diamine (IAEDANS) and subsequent peptide mapping procedure as described (Tatsumi et al., 1992). The far-UV circular dichroism (CD) spectrum was recorded at 0.2 mg/ml of protein as described by Takahashi et al. (Takahashi et al., 1996). The fluorescence spectrum of ovalbumin was measured with a fluorescence spectrophotometer (model F-3000, Hitachi). The intrinsic tryptophan residues in ovalbumin were excited at 295 nm, and the emission spectrum was recorded in a wavelength range from 300 to 420 nm at a constant temperature of 25 °C.

Analysis of disulfide formation in periplasmic space.

For analysis of the intrachain disulfide formation in the periplasm, the alkylated periplasmic fraction and purified recombinant ovalbumin that had been incubated with 50 mM monoiodoacetic acid in a 30 mM Tris-HCl buffer (pH 8.0) for 15 min at 25 °C were mixed with 0.33 volume of an SDS-buffer (0.25 M Tris-HCl at pH7.0 containing 4% SDS and 40% glycerol). Prior to SDS-PAGE, the protein samples were incubated with or without 5% 2-mercaptoethanol in boiling water for 2 min.

Results

To investigate the secretion of recombinant ovalbumin into periplasmic space, the samples fractionated by osmotic shock treatments were analyzed by western blotting using the ovalbumin specific antiserum following to SDS-PAGE. The visualized bands were quantified using the flatbed scanner and the software. The accuracy of the fractionation was confirmed by the maker enzyme assays. The periplasmic fractions were little detected the β-galactosidase activity (Table 1).

Table 1 Cellular distribution of recombinant ovalbumin in *E. coli*.[a]

	Alkaline phosphatase unit (%)	β-galactosidase unit (%)	Recombinant protein mg (%)
Wild			
Periplasm	0.51 (98.1)	5.7 (7.3)	32.3 (24.7)
Cytoplasm	0.01 (1.9)	72.9 (92.7)	76.2 (58.3)
Debris	— (—)	— (—)	22.2 (7.0)
Δ1-39			
Periplasm	0.31 (93.9)	0.7 (1.3)	0.0 (0.0)
Cytoplasm	0.02 (6.3)	54.1 (98.7)	0.8 (0.7)
Debris	— (—)	— (—)	118.7 (99.3)

[a] The enzyme activities and the amounts of recombinant ovalbumin are expressed as the values corresponding to one liter of the original unfractionated culture.

The secretion of wild type into the periplasmic space in *E. coli* cells was carried out and the ratio of recombinant protein in the periplasmic fraction showed about 25% of expressed proteins. While Δ1-39 mutant, which was truncated most of the internal signal sequence, was localized in debris (Table 1).

Egg white ovalbumin contains four cysteine residues (Cys11, Cys30, Cys367, and Cys382) and one cystine disulfide (Cys73-Cys120) in a single polypeptide chain of 385 amino acid residues (Forthergill & Forthergill, 1970). To investigate whether or not the native disulfide bond was formed in wild type, the purification of wild type from the periplasmic fraction was carried out. The purified recombinant protein was analyzed for its N-terminal sequence. The recombinant protein consisted of two sequences: Gly-Ser-Ile-Gly- and Met-Gly-Ser-Ile-. The first sequence is consistent with the N-terminus of egg white ovalbumin, except for the lack of N-terminal acetylation, while the second sequence has an additional N-terminal Met residue over egg white ovalbumin. The results of CD- and fluorescence spectra indicated the almost same conformation as that of the egg white protein (data not shown). The purified protein was analyzed for the intrachain disulfide formation by peptide mapping approach that has been established (Tatsumi et al., 1992). This approach utilizes a cystein-labeling technique with a fluorescent alkylation reagent and allows quantitative determination of the disulfide-forming cystine residues. As shown in Fig. 1, the ratios of the observed labeling values for Cys73and Cys120, relative to the overall labeling values, were almost 0.8 for the recombinant protein, while the labeling values were less than 0.12 for the other four cysteine residues. These data demonstrate that most, if not all, of the recombinant protein molecules contained the native disulfide bond, Cys73-Cys120.

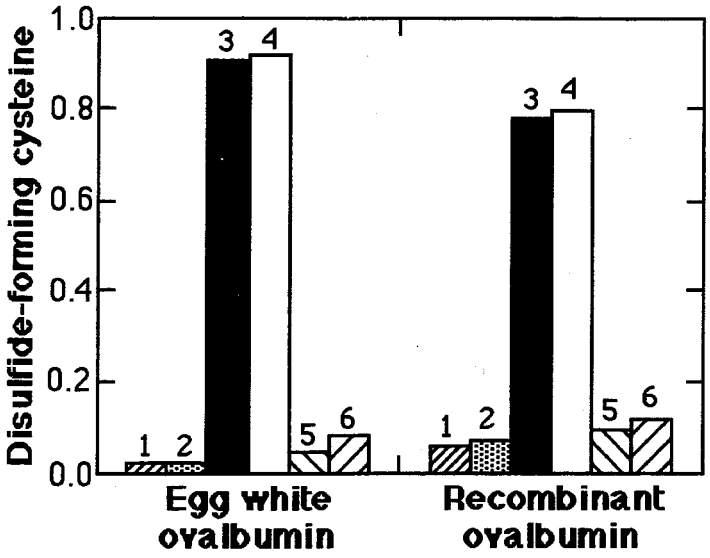


Fig. 1. Analysis of disulfide-forming cysteine residues in the purified recombinant ovalbumin. The purified recombinant ovalbumin and the egg white protein control were analyzed for their intrachain disulfide bond formation by a peptide mapping analysis. After cysteine sulfhydryls had been alkylated with iodoacetamide, protein disulfide bonds were reduced with DTT and labeled with IAEDANS. The protein samples were extensively digested with proteases and analyzed to identify fluorescent peptides by reversed-phase HPLC as described in the text. For the overall labeling experiments, the intact ovalbumin was fully reduced with DTT, and all cysteine residues were labeled with IAEDANS and proteolyzed in the same way. The numbers 1, 2, 3, 4, 5, and 6 on the tops of the bars represent, respectively, the cysteine residues of Cys11, Cys30, Cys73, Cys120, Cys376, and Cys382. The disulfide-forming cysteines are expressed as the labeling data in the sample run divided by those in the overall labeling run.

It was investigated whether or not the recombinant protein contained the native disulfide bond in the periplasmic space of the *E. coli* cell. Ovalbumin exerts a slightly greater mobility in the disulfide-bonded form than in the disulfide-reduced form (Tatsumi et al., 1992). The recombinant protein in the periplasmic fraction that had been alkylated immediately after osmotic

shock treatment was analyzed by SDS-PAGE under disulfide-reduced and non-reduced conditions. The purified recombinant protein with the native disulfide was also analyzed in the same way after alkylation with monoiodoacetic acid. As shown in Fig. 2, the purified protein migrated with greater mobility in the non-reduced condition than in the disulfide-reduced condition (lanes 1 and 3). This is consistent with the presence of the intrachain disulfide bond in the purified recombinant protein. In contrast, the periplasmic protein showed the same lower mobility in the disulfide-reduced and non-reduced conditions (lanes 2 and 4). It is therefore very likely that most of the recombinant protein did not form an intrachain disulfide bond in the periplasm.

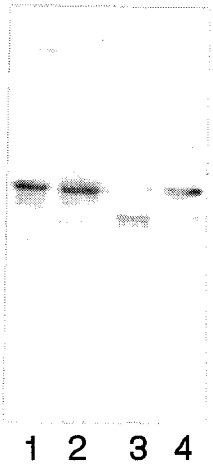


Fig. 2. Analysis of the intrachain disulfide formation in the periplasmic space. The purified recombinant protein (lanes 1 and 3) was incubated with monoiodoacetic acid and analyzed by SDS-PAGE and subsequent western blotting as described in the text. The recombinant ovalbumin in the cytoplasmic fraction (lanes 2 and 4) that had been alkylated immediately after cellular disruption was also analyzed in the same way. Prior to the electrophoresis, the protein samples were pretreated with 2-mercaptoethanol in a boiling water bath for 2 min (lanes 1 and 2) or not pretreated (lanes 3 and 4).

Discussion

Ovalbumin has been shown to be secreted utilizing an internal signal, which is localized within a hydrophobic region comprising the first 22 to 41 amino-terminal residues, by secretion analyses using a *Xenopus* oocyte expression system (Tabe et al., 1984). In previous reports, ovalbumin synthesized in *E. coli* has been indicated the possibility of the secretion into periplasmic space utilized the signal sequence (Fraser & Bruce, 1978; Mercereau-Puijalon et al., 1978). The expression plasmid has, however, included 19 residues of N-terminal extension that are derived from β-galactosidase (Fraser & Bruce, 1978) or the loss of four residues of the original N-terminus of ovalbumin as well as the extension of nine residues from β-galactosidase sequence (Mercereau-Puijalon et al., 1978). As shown in Table 1 and N-terminal analysis, the full length of ovalbumin, which is included no extension, was secreted into the periplasmic space in *E. coli* cell and the N-terminal region was not cleaved. While above a half of recombinant protein remained in the cytoplasm (Table 1). The lower efficiency would be caused as a result that the amount of expressed protein goes up to over the secretory ability in *E. coli* cell. On the other hand, most of Δ1-39 mutant was localized in debris (Table1). This data shows that the N-terminal region containing the signal sequence was required to sustain the ternary structure of ovalbumin. According to the X-ray crystallographic study (Wright et al., 1990; Stein et al., 1991), the internal signal of ovalbumin is sequestered from solvent in the native structure (Fig. 3). This suggests the possibility for a correct folding pathway of ovalbumin *in vivo* in which the initially membrane-inserted signal sequence is later buried into the succeeding segments upon its release from the membrane.

Egg white ovalbumin contains four cysteine residues (Cys11, Cys30, Cys367, and Cys382) and one cystine disulfide (Cys73-Cys120) in a single polypeptide chain of 385 amino acid residues

Fig. 3 Schematic view of ovalbumin. The figure is based on the X-ray crystallographic data of ovalbumin (Stein et al., 1991) and was drawn using the MolScript program (Kraulis, 1991). The internal signal sequence is colored black. The number show the number of amino acid residue.

(Nisbest et al., 1981). It is interesting that this protein takes a compact and native-like conformation in its disulfide -reduced state *in vitro* (Takahashi et al., 1991) and that four cysteine residues do not form the disulfide bond in a molecule. These phenomenon indicate the possibility that the formation is carried out after the protein folding following to the secretion. As shown in Table 1 and Fig. 2, the secretion and folding of recombinant protein were carried out without the formation of disulfide bond. As shown in Fig.2, the formation of the disulfide bond

is not carried out in the cellular environment. On the other hand, the purified protein, which was contact with the oxygen during the purification, contained the native disulfide bond (Fig. 1). The presence of the correct disulfide bond in the purified recombinant ovalbumin can, therefore, be accounted for by specific cysteine oxidation with oxygen from the air during the isolation procedure. These results show the possibility that the formation of disulfide bond in the ovalbumin molecules folded correctly is carried out after ovalbumin was secreted into the extracellular space, such as egg white. Recent studies have showed that hen egg white has a homodimeric sulfhydryl oxidase (Hoober et al., 1996) and the enzyme catalyzes the oxidation of sulfhydryl groups to disulfides with reduction of oxygen to hydrogen peroxide (Hoober & Thorpe, 1999). The presence of this enzyme suggests strongly our possibility. Furthermore, an average laying hen produces one egg containing about 2 g of ovalbumin every 26 hours (Palmiter, 1975). The rate in estrogen-primed chicks is about 6 x 10^5 molecules/min/tubular gland cell (Palmiter, 1975). The large amounts of production causes evolutionary the absence of a clearable prepiece, which would be advantage to the cell (von Heijne, 1980). In a similar manner, the disulfide formation in extracellular space may be also advantage to the cell.

CHAPTER 2.

Structural properties of recombinant ovalbumin and its thermostabilization by alkaline treatment.

Ovalbumin in stored shell eggs is transformed into the more stable form, S-ovalbumin, accompanying a rise in the pH value of egg white during storage (Smith & Back, 1965). The presence of S-ovalbumin can be clearly detected by differential scanning calorimetry (DSC) of egg white (Donavan & Mapes, 1975). The denaturation temperature of S-ovalbumin is about 8 °C higher than that of native ovalbumin (Donavan & Mapes, 1975). Such a difference may affect the food functionalities of egg white such as its coagulating and foaming properties; for example, the increased thermostability is likely to account for the low rigidity of heat-induced gels made from S-ovalbumin (Egelandsdal, 1986). The transformation mechanism for ovalbumin into S-ovalbumin is of interest in food science as well as in protein science.

Isolated ovalbumin can be also transformed into S-ovalbumin by an alkaline treatment (Smith & Back, 1965). The structural characteristics of alkaline-induced S-ovalbumin have been extensively studied. S-ovalbumin and ovalbumin have been shown to have different amide contents (Nakamura et al., 1981; Kato et al., 1986) and hydrodynamic volume (Nakamura & Ishimaru, 1981). The slightly increased β-sheet and decreased α-helix contents in S-ovalbumin have been also shown (Kint & Tomimatsu, 1979; Huntigton et al., 1995). Despite these extensive structural comparisons, the molecular mechanism for S-ovalbumin formation is still not well understood.

As an alternative approach, the use of a covalent derivative of ovalbumin may provide useful information about the mechanism. Ovalbumin comprises of a single polypeptide chain of 385

amino acid residues (Nisbet et al., 1981) which has a single carbohydrate chain linked covalently with Asn292 (Glabe et al., 1980). The protein has a single disulfide between Cys73 and Cys120 and four free sulfhydryl groups, Cys11, Cys30, Cys376 and Cys382 (Fothergill & Fothergill, 1970). On the basis of the different contents of phosphate groups, it is classified into the three molecular species of A_1-, A_2-, and A_3-ovalbumin, which respectively contain two, one, and no phosphate group in each molecule (Taborsky, 1974). The limited proteolysis of ovalbumin with subtilisin yields an ovalbumin derivative named plakalbumin (Ottesen, 1958), which lacks a hexapeptide (Ala347-Ala352) in the loop region. By an alkaline treatment, plakalbumin has been shown to be converted to a more thermostable form, indicating that destruction of the covalent structure around the hexapeptide did not affect the stability (Shitamori & Nakamura, 1983). It has been previously shown that the transformation of ovalbumin into S-ovalbumin required the presence of the native disulfide bond, Cys73-Cys120 (Takahashi et al., 1996).

To investigate in more detail the correlation between the covalent structure and S-ovalbumin formation, the use of recombinant ovalbumin would be crucial. Takahasi et al (1995) has previously established an *E. coli* expression system for ovalbumin. In the present study, recombinant ovalbumin was purified from the cytoplasm of *E. coli*, and its structural characteristics were compared with those of egg white ovalbumin. The conformation and disulfide structure of the recombinant protein were essentially the same as those of egg white protein. However, none of the post-translational modifications known in egg white ovalbumin, including N-terminal acetylation, phosphorylation, and glycosylation, were detected in the recombinant protein. The recombinant protein was transformed into a more thermostable form by an alkaline treatment. These data are consistent with the view that the post-translational modifications are not related to the transformation of ovalbumin into S-ovalbumin.

Materials and Methods

Materials.

Ovalbumin was purified from fresh egg white as described in CHAPTER 1. This ovalbumin preparation was used for the analysis of post-translational modifications (see Fig. 8). The protein was further purified by anion-exchange chromatography (Kitabatake et al., 1988a), and the obtained A_1-ovalbumin was used for the other analyses. IAEDANS was purchased from Aldrich Co. Other chemicals, including DTT, urea, and IAA, were of guaranteed grade from Nacalai Tesque. Trypsin (type XI), chymotrypsin (type II), and acid phosphatase (EC 3.1.3.2) were purchased from Sigma. Achromobacter protease I (EC 3.4.21.50) was obtained from Wako Pure Chemical Industries.

Production of ovalbumin in E. coli, cellular fractionation, and protein purification.

E. coli BL21 (DE3) cells harboring the expression vector, pET/Ova (Takahashi et al., 1995), were inoculated into 4 ml of a LB medium supplemented by 50 μg/ml of ampicillin and incubated overnight at 37 °C while agitating. This culture was then transferred into two shaking flasks each containing 1 liter of the LB medium supplemented by the same concentration of ampicillin and incubated in a same way for 16 h. The periplasmic and cytoplasmic fractions were obtained after an osmotic shock treatment of the cells (Neu & Heppel, 1965): The *E. coli* cells collected by centrifugation (8,000 x g, 5 min) from the 2-liter culture were suspended in 100 ml of a 50 mM Na-phosphate buffer (pH 6.0) containing 20 % sucrose and 1 mM Na-EDTA, incubated at 25 °C for 20 min, and centrifuged. The precipitated cells were resuspended in 100 ml of cold distilled water, incubated at 0 °C for 20 min, and centrifuged. The supernatant was stored as the periplasmic

fraction. The precipitated cells were suspended in 100 ml of the 50 mM Na-phosphate buffer (pH 6.0) containing 1.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na-EDTA, disrupted with a sonicator (250 Sonifier, Branson) for 1 min 20 times at 0 °C, and then centrifuged. The clear supernatant was used as the cytoplasmic fraction. To analyze the intrachain disulfide formation in the cytoplasm, the precipitated cells after the distilled cold water treatment were suspended in a 30 mM Tris-HCl buffer (pH 8.0) containing 1.5 mM PMSF, 1 mM Na-EDTA, and 50 mM monoiodoacetic acid, disrupted, and then centrifuged in the same way, before the supernatant was incubated for 15 min at 25 °C. For marker enzyme assays (β-galactosidase for cytoplasm (Craven et al., 1965) and alkaline phosphatase for periplasm (Garen & Levinthal, 1960)), 0.125 ml of the fractions were employed.

Purification of the recombinant ovalbumin was accomplished by concentrating the cytoplasmic proteins corresponding to 1.8 liter of the original culture of pET/Ova harboring cells by precipitation in an ammonium sulfate solution (90% saturation) and subsequently dissolving in a 10 mM Na-phosphate buffer at pH 6.0. The protein samples were dialyzed against the same buffer, applied to an anion-exchange column (HiLoad 26/10 Q-Sepharose HP, Amersham Pharmacia Biotech.) and eluted by a linear 0.01 to 0.15 M gradient of the Na-phosphate buffer at pH 6.0. Ovalbumin peaks were collected. The cytoplasmic protein was further purified by using another anion-exchange column (Mono Q HR 5/5, Amersham Pharmacia Biotech), the protein sample being applied to the column and then eluted by the same linear gradient of the Na-phosphate buffer. The purified proteins were concentrated by precipitation in an ammonium sulfate solution in the same way. The protein concentration was estimated as described in CHAPTER 1.

SDS-PAGE.

SDS-PAGE was carried out as described in CHAPTER 1. Proteins were stained with 0.25% Coomassie Brilliant Blue R-250 or were made visible by a western blotting analysis, using the ovalbumin specific antiserum as described previously (Takahashi & Hirose, 1992) To analyzed the intrachain disulfide formation in the cytoplasm, the alkylated cytoplasmic fraction and purified recombinant ovalbumin that had been incubated with 50 mM monoiodoacetic acid in a 30 mM Tris-HCl buffer (pH 8.0) for 15 min at 25 °C were mixed with 0.33 volume of an SDS-buffer (0.25 M Tris-HCl at pH7.0 containing 4% SDS and 40% glycerol). Prior to SDS-PAGE, the protein samples were incubated with or without 5% 2-mercaptoethanol in boiling water for 2 min.

Chemical analyses.

The N-terminal amino acid sequence was determined as described in CHAPTER 1. To analyze the amino acid composition, the purified recombinant protein, with or without preincubation with 5 mM DTT in 8 M urea and a 50 mM Tris-HCl buffer at pH 8.2 and 37 °C for 10 min, was alkylated by incubating with 30 mM monoiodoacetic acid at 37 °C for 10 min, and then hydrolyzed in 6 N HCl containing 1% (v/v) phenol at 110 °C for 20 h. The amino acid composition was determined with an amino acid analyzer (L-8500A, Hitachi).

The disulfide-forming half-cystines were identified by protein alkylation with a fluorescent reagent, IAEDANS and subsequent peptide mapping as described (Tatsumi et al., 1994).

Spectroscopic analysis.

The far-UV CD spectrum was recorded at 0.2 mg/ml of protein as described by Takahashi et al. (1996). The fluorescence spectrum of ovalbumin was measured as described in CHAPTER 1.

Assay for the carbohydrate chain.

Egg white ovalbumin (0.25 μg) and the recombinant protein (0.5 μg) were subjected to SDS-PAGE (10% polyacrylamide) and blotted onto a nitrocellulose membrane. Labeling and detection of total carbohydrate was done with a glycoprotein detection system (ECL glycoprotein detection system, Amersham Pharmacia Biotech.) and exposed to an X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech.).

Assay for the phosphate group with acid phosphatase.

Phosphorylation in the recombinant protein was detected as described by Kitabatake et al. (1988a). The recombinant protein and egg white protein control were incubated at 0.14 mg/ml with 7.8 or 78 μg/ml of acid phosphatase (0.005 or 0.05 unit/ml) at 37 °C for 20 h in a 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.5) containing 0.02% NaN_3. The proteins in 15 μl of the reaction mixture were subjected to native PAGE (9% polyacrylamide) according to the standard method by Davis (1964) and stained with 0.25% Coomassie Brilliant Blue R-250.

Differential scanning calorimetry (DSC).

The disulfide bonds in the recombinant ovalbumin and in the egg white protein control were reduced by incubating with 15 mM DTT in a Tris-HCl buffer at pH 8.0 and 37 °C for 2 h. The disulfide-reduced proteins, as well as the non-reduced proteins, were passed through a Sephadex

column (NAP-10, Amersham Pharmacia Biotech.) that had been equilibrated with a 10 mM Na-phosphate buffer at pH 6.0. Transformation of the recombinant protein into the thermostable form was carried out by incubating the protein solution in a 0.1 M glycine buffer at pH 9.9 and 55 °C for 16 h (Smith & Back, 1965) and then passing through the Sephadex column in the same way. The thermostability of the ovalbumin samples was analyzed with a DSC (Micro Cal). The protein concentration was 0.2 mg/ml in the 10 mM Na-phosphate buffer at pH 6.0. The temperature was scanned at 1 $K \cdot min^{-1}$.

Results

Purification of the recombinant ovalbumin from the cytoplasmic fraction

In previous studies, some molecular properties have been analyzed for recombinant ovalbumin that had been purified from total *E. coli* cellular extracts (Takahashi et al., 1995). The recombinant protein has, however, been found to be produced in both the periplasm and cytoplasm as shown in Table 1. In this chapter, therefore, recombinant ovalbumin was purified from the cytoplasm of *E. coli* cells. The periplasmic and cytoplasmic fractions were obtained after the osmotic shock treatment (Neu & Heppel, 1965) of the ovalbumin-harboring cells and were subjected to the cellular marker enzyme assays. As shown in Table 1, periplasm-specific alkaline phosphatase activity, calculated as the enzyme activity per ml of the culture, was 0.51 unit for the periplasmic fraction, while it was only 0.01 unit for the cytoplasmic fraction. In contrast, for the cytoplasmic marker enzyme, β-galactosidase, 5.7 and 72.9 unit activities were detected in the periplasmic and cytoplasmic fractions, respectively. The results from marker enzyme assays revealed that the cellular fractionation had worked correctly.

The protein in the cytoplasmic fraction was analyzed by SDS-PAGE (Fig. 4) and detected with Coomassie Brilliant Blue (Fig. 4, lane 1). When the protein bands were made visible by the western blotting procedure, using the ovalbumin-specific antiserum, recombinant ovalbumin was detected (Fig. 4, lane 3). As a prerequisite step in the structural analysis of recombinant ovalbumin, the cytoplasmic fraction was subjected to purification. The cytoplasmic proteins corresponding to 1.8 liter of the original culture were purified by anion-exchange column chromatography. The cytoplasmic protein was further purified by using another anion-exchange column. The purified recombinant ovalbumin sample was found to be almost homogeneous (Fig. 4, lane 5).

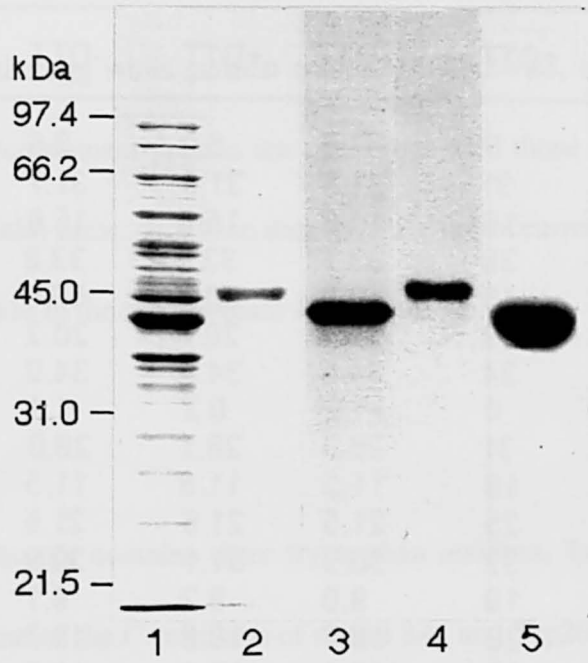


Fig. 4. Purification of Recombinant Ovalbumin in the Cytoplasm of *E. coli*. Proteins corresponding to 12.5 μl of the cytoplasmic fraction (lane 1), 1.0 μg of egg white ovalbumin (lane 2), and 3.3 μg of purified recombinant ovalbumin (lane 5) were electrophoresed on SDS polyacrylamide gel (10% polyacrylamide) and stained with Coomassie Brilliant Blue R-250 as described in the text. The western blotting analysis was carried out by using 2.5 μl of the cytoplasmic fraction (lane 3) and 0.1 μg of egg white ovalbumin (lane 4) as described in the text. On the left side, the migration of phosphorylase B (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa) are shown.

Chemical structures of the recombinant ovalbumin

The purified recombinant ovalbumin was analyzed for its N-terminal sequence. The recombinant protein consisted of two sequences: Gly-Ser-Ile-Gly- and Met-Gly-Ser-Ile-. The first sequence is consistent with the N-terminus of egg white ovalbumin, except for the lack of N-terminal acetylation, while the second sequence has an additional N-terminal Met residue over egg white ovalbumin.

Table 2. Amino Acid Composition of the Recombinant Ovalbumin

	Molar ratio[a] (residue/protein)					
	Theoretical		Recombinant		Egg white	
	-DTT[b]	+DTT	-DTT	+DTT	-DTT	+DTT
SCMC[c]	4	6	3.5	6.0	3.6	6.1
Asx	31	31	31.6	31.8	31.7	30.8
Thr	16	16	15.6	15.8	15.6	14.3
Ser	38	38	33.7	33.9	33.8	33.1
Glx	48	48	49.9	50.2	50.0	49.1
Gly	19	19	20.1	20.7	20.2	19.1
Ala	34	34	34.0	34.0	34.0	34.0
Cystine	1	0	1.2	0.2	1.1	0.2
Val	31	31	28.0	28.1	28.0	27.4
Met	16	16	11.5	11.8	11.5	12.0
Ile	25	25	21.5	21.6	21.5	20.7
Leu	32	32	30.9	31.1	30.9	29.8
Tyr	10	10	9.0	9.2	9.1	8.3
Phe	20	20	18.7	18.9	18.7	18.3
Lys	20	20	18.9	19.2	18.9	18.5
His	7	7	8.4	8.6	8.3	7.0
Arg	15	15	14.7	14.7	14.6	14.5
Pro	14	14	14.9	14.9	14.8	14.5

[a]Data were estimated by normalizing to the theoretical value for Ala.

[b]DTT is dithiothreitol. +DTT and -DTT represent the ovalbumin samples, respectively, with and without incubation in the presence of DTT before alkylation by monoiodoacetic acid and subsequent analysis for the amino acid composition.

[c]SCMC represents S-carboxymethyl cysteine.

The amino acid composition of the recombinant protein was determined and compared with that of the egg white protein. To clearly determine the contents of cystine and cysteine residues, the author employed the ovalbumin samples with or without pre-treatment with DTT for subsequent alkylation with monoiodoacetic acid. As summarized in Table 2, the number of S-carboxy-methyl cysteine residues was almost consistent with the theoretical value of six for the recombinant protein that had been pretreated with DTT, while the number was about four for the protein without pre-treatment with the thiol. In addition, the number of cystine residues was 1.2 for the recombinant protein without the DTT pre-treatment, while little evidence of a cystine residue was detected for the thiol-treated protein. These cystine and cysteine profiles are almost the same as those of the egg white protein control. Likewise, the numbers of the other amino acid residues in the recombinant protein are consistent with those of egg white ovalbumin within the range of experimental error. These data indicate the occurrence of four cysteine sulfhydryls and one cystine disulfide in the recombinant ovalbumin.

Optical properties

Egg white ovalbumin contains three tryptophan residues, Trp148 in helix F, Trp184 as the nearest neighbor residue of the C-terminus of strand 3A, and Trp267 in helix H (Stein et al., 1991). The conformational states of the recombinant proteins were analyzed by the intrinsic tryptophan fluorescence spectrum. As shown in Fig. 5, the fluorescence emission spectrum of the recombinant protein was similar to the spectrum of egg white ovalbumin that displayed an emission maximum at 338 nm.

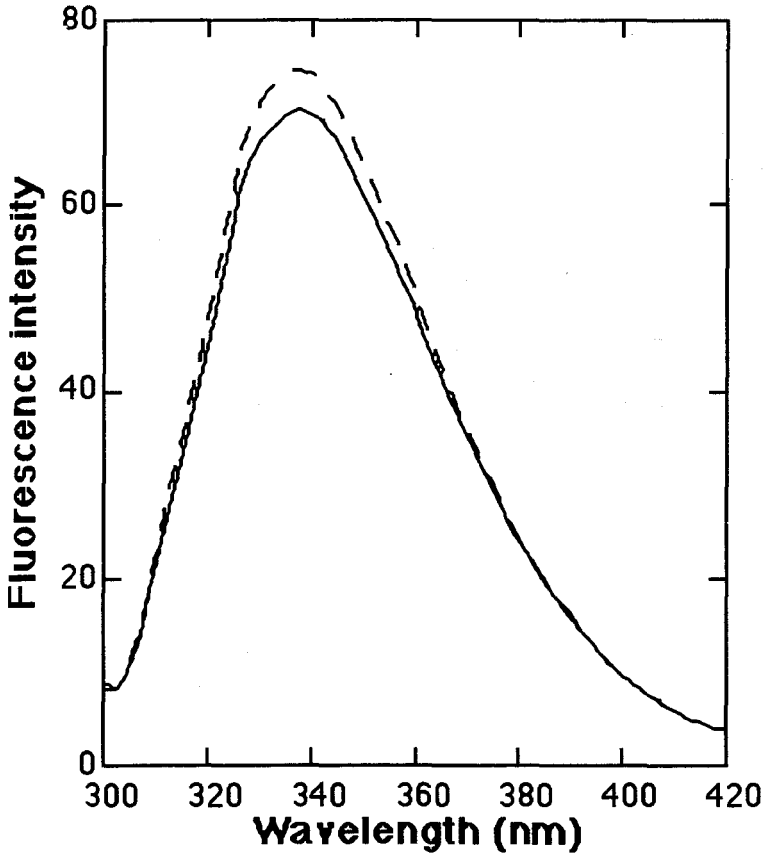


Fig. 5. Intrinsic Tryptophan Fluorescence Spectra. The tryptophan residues in recombinant ovalbumin (solid line) and in egg white ovalbumin (dashed line) were excited at 295 nm, and the emission spectra were recorded at 25 °C. The protein concentration was 0.05 mg/ml in a 50 mM Tris-HCl buffer at pH 7.5 containing 1 mM Na-EDTA.

The far-UV CD spectrum is reliable for evaluating the secondary structure of ovalbumin, since the α-helix content estimated by this optical method in egg white ovalbumin (30%) (Gorbanoff, 1969; Batra et al., 1990) is almost exactly the same as the content (30.6%) determined by X-ray crystallography (Stein et al., 1991) It has been shown that recombinant ovalbumin purified from whole *E. coli* cell displayed almost the same far-UV CD spectrum as that of egg

white ovalbumin. The author reconfirmed the same far-UV CD spectra for the cytoplasmic recombinant ovalbumin and egg white ovalbumin (data not shown), indicating that the recombinant protein had essentially the same secondary structure as that of egg white ovalbumin.

Peptide mapping approach

Egg white ovalbumin contains four cysteine residues (Cys11, Cys30, Cys367, and Cys382) and one cystine disulfide (Cys73-Cys120) in a single polypeptide chain of 385 amino acid residues (Nisbet et al., 1981). The disulfide-forming cysteine residues in the recombinant protein were analyzed by the peptide mapping approach that had been previously established (Tatsumi et al, 1994). This approach utilizes a cysteine-labeling technique with a fluorescent alkylation reagent and allows quantitative determination of the disulfide-forming cysteine residues. As shown in Fig. 6, the ratios of the observed labeling values for Cys73 and Cys120, relative to the overall labeling values, were almost 0.8 or more for the recombinant protein, while the labeling values were less than 0.1 for the other four cysteine residues. These data demonstrate that most, if not all, of the recombinant protein molecules contained the native disulfide bond, Cys73-Cys120.

Analysis for intrachain disulfide formation in the cytoplasm

It was investigated whether or not the native disulfide bond was formed in the cytoplasm of the *E. coli* cell. Ovalbumin exerts a slightly greater mobility in the disulfide-bonded form than in the disulfide-reduced form (Tatsumi et al., 1994). The recombinant protein in the cytoplasmic fraction that had been alkylated immediately after cellular disruption was analyzed by SDS-PAGE under disulfide-reduced and non-reduced conditions. The purified recombinant protein with the native disulfide was also analyzed in the same way after alkylation with monoiodoacetic acid.

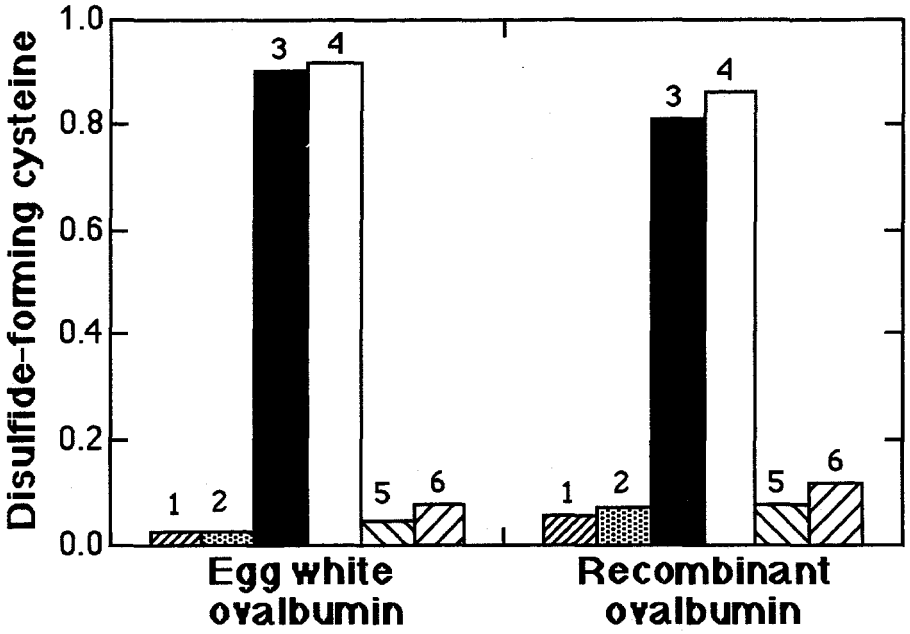


Fig. 6. Analysis of Disulfide-forming Cysteine Residues in Recombinant Ovalbumin. The recombinant ovalbumin and the egg white protein control were analyzed for their intrachain disulfide bond formation by a peptide mapping analysis. After cysteine sulfhydryls had been alkylated with iodoacetamide, protein disulfide bonds were reduced with DTT and labeled with IAEDANS. The protein samples were extensively digested with proteases and analyzed to identify fluorescent peptides by reversed-phase HPLC as described in the text. For the overall labeling experiments, the intact ovalbumin was fully reduced with DTT, and all cysteine residues were labeled with IAEDANS and proteolyzed in the same way. The numbers 1, 2, 3, 4, 5, and 6 on the tops of the bars represent, respectively, the cysteine residues of Cys11, Cys30, Cys73, Cys120, Cys376, and Cys382. The disulfide-forming cysteines are expressed as the labeling data in the sample run divided by those in the overall labeling run.

As shown in Fig. 7, the purified protein migrated with greater mobility in the non-reduced condition than in the disulfide-reduced condition (lanes 1 and 3). This is consistent with the presence of the intrachain disulfide bond in the purified recombinant protein. In contrast, the cytoplasmic protein showed the same lower mobility in the disulfide-reduced and non-reduced conditions (lanes 2 and 4). It is therefore very likely that most of the recombinant protein did not form an intrachain disulfide bond in the cytoplasm.

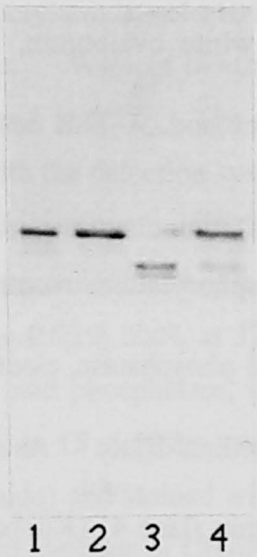


Fig. 7. Analysis of the Intrachain Disulfide Formation in the Cytoplasm. The purified recombinant protein (lanes 1 and 3) was incubated with mono-iodoacetic acid and analyzed by SDS-PAGE and subsequent western blotting as described in Material and methods of CHAPTER 2. The recombinant ovalbumin in the cytoplasmic fraction (lanes 2 and 4) that had been alkylated immediately after cellular disruption was also analyzed in the same way. Prior to the electrophoresis, the protein samples were pretreated with 2-mercapto-ethanol in a boiling water bath for 2 min (lanes 1 and 2) or not pretreated (lanes 3 and 4).

Absence of post-translational modifications

Egg white ovalbumin is a glycoprotein with a single carbohydrate chain and consists of the three protein species of A_1-, A_2-, and A_3-ovalbumin, which contain two, one, and no phosphate group per molecule, respectively. To examine whether or not these modifications were included in the recombinant ovalbumin, the purified protein was analyzed by PAGE. For glycoprotein detection, the polyacrylamide gel after SDS-PAGE was blotted onto a membrane, and the protein bands were detected by using the ovalbumin-specific antiserum or by a carbohydrate labeling method. As shown in Fig. 8A, both the egg white and recombinant proteins were detected by the western blotting analysis, while egg white ovalbumin, but not recombinant ovalbumin, was detected by the carbohydrate labeling method. This demonstrates that the recombinant protein was not glycosylated.

For the analysis of protein phosphorylation, recombinant ovalbumin and the egg white protein control were incubated with acid phosphatase, electrophoresed on a native polyacrylamide gel, and then stained with Coomassie Brilliant Blue. As shown in Fig. 8B, the egg white protein largely consisted of A_1- and A_2-ovalbumin (lane 4) (Kitabatake et al., 1988a). Incubation with acid phosphatase transformed the protein sample by dephosphorylation into one consisting of A_2- and A_3-ovalbumin (lanes 5 and 6). In contrast, the mobility of the recombinant protein was not changed by incubating with the same concentrations of the enzyme (lanes 1, 2, and 3). These results indicate that the recombinant protein did not contain a phosphate group in the molecule.

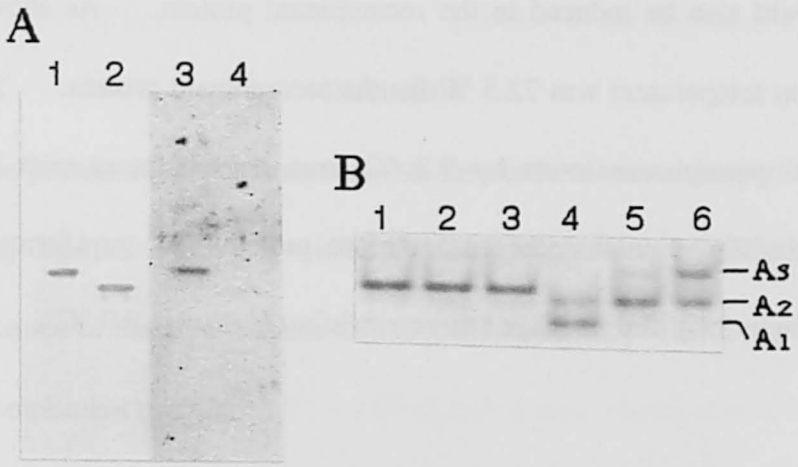


Fig. 8. Analyses of the Post-translational Modifications. In panel A, egg white ovalbumin (lanes 1 and 3) and recombinant ovalbumin (lanes 2 and 4) were electrophoresed on SDS polyacrylamide gel (10% polyacrylamide) and blotted onto a nitrocellulose membrane. Western blotting was carried out by using the anti-ovalbumin antiserum (lanes 1 and 2). The carbohydrate was detected by a chemical labeling procedure with the detection system described in the text (lanes 3 and 4). In panel B, recombinant ovalbumin (lanes 1, 2, and 3) and egg white ovalbumin (lanes 4, 5, and 6) were incubated at 0.14 mg/ml for 20 h in a 50 mM MES buffer (pH 5.5) containing 0.02% NaN_3 at 37 °C with 7.8 (lanes 2 and 5) or 78.0 (lanes 3 and 6) μg/ml of acid phosphatase, or without the enzyme (lanes 1 and 4). The proteins (2.0 μg) in 15 μl of the reaction mixture were subjected to native PAGE (9% polyacrylamide) and stained with Coomassie Brilliant Blue R-250 as described in Material and methods of CHAPTER 2.

Thermostability

Previous reports have shown that the thermostability of ovalbumin, as evaluated by DSC was altered in modified protein derivatives such as the disulfide-reduced form (Takahashi et al, 1991) and S-ovalbumin (Donavan & Mapes, 1975). As control experiments, the author confirmed under the present conditions that egg white ovalbumin (A_1-ovalbumin) displayed the denaturation tempera-ture lower by 6.5 °C upon disulfide reduction but higher by 8.5 °C upon transformation into S-ovalbumin (Fig. 9A). The author investigated whether or not such alterations in the

thermostability would also be induced in the recombinant protein. As shown in Fig. 9B, the thermal denaturation temperature was 72.5 °C for the recombinant protein. The thermostability of the recombinant protein was lower by 7.2 °C from the treatment with DTT. The most important feature in Fig. 9 is that the recombinant protein was transformed by the alkaline treatment into a protein form that displayed thermostability higher by 8.7 °C.

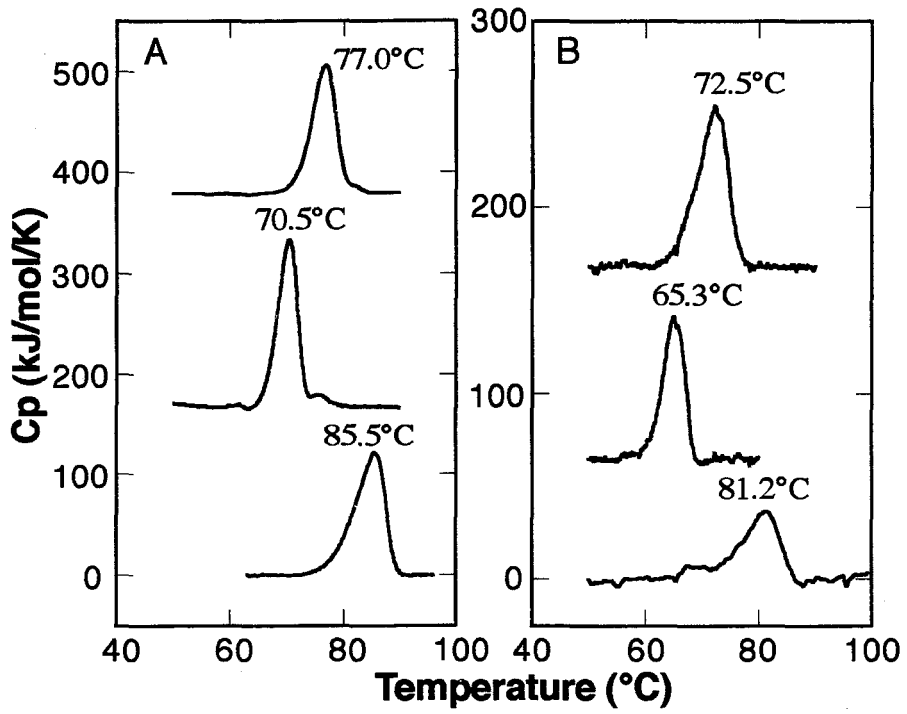


Fig. 9. Thermostability of Different Ovalbumin Forms. The thermostability of the disulfide-bonded (top), disulfide-reduced (middle), and alkali-treated (bottom) forms of egg white ovalbumin (panel A) were analyzed with a DSC. The temperature was scanned at 1 $K \cdot min^{-1}$ and the protein concentration was 0.2 mg/ml in a 10 mM Na-phosphate buffer at pH 6.0. Recombinant ovalbumin (panel B) was analyzed in the same way. Endothermic transition profiles have been arbitrarily shifted on the ordinate scale for clarity.

Discussion

The data in the present report demonstrate that the conformational properties and polypeptide structures, including disulfide paring, are indistinguishable between egg white and recombinant ovalbumin, but that none of the post-translational modifications known with egg white protein were detectable in the recombinant protein.

Recent genetic and biochemical studies have revealed that a protein disulfide bond is formed in the bacterial periplasm utilizing the disulfide-forming protein system, including Dsb A, Dsb B, and Dsb C (Akiyama et al., 1992; Bardwell et al., 1993; Missiaks et al., 1994). In this context, the correct disulfide formation in purified recombinant ovalbumin from the cytoplasmic fraction (Table 2; Fig. 6) appears unusual. However, the results from the SDS-PAGE analysis (Fig. 7) strongly suggest that the recombinant ovalbumin in the bacterial cytoplasm was in the disulfide-reduced form. Ovalbumin assumes a native-like conformation without any disulfide bond formation (Takahashi et al., 1992), and the disulfide-reduced form can be readily transformed into the native disulfide form under oxidative conditions (Takahashi et al., 1991). The presence of the correct disulfide bond in the purified recombinant ovalbumin can, therefore, be accounted for by specific cysteine oxidation with oxygen from the air during the isolation procedure.

The denaturation temperature of the recombinant protein is lower by 4 to 5 °C than of the egg white protein (A_1-ovalbumin) in either the disulfide-bonded, disulfide-reduced, or alkali-treated form (Fig. 9). A_1-ovalbumin contains two phosphate groups in a molecule (Taborsky, 1974) and is highly glycosylated (Glabe et al., 1980); the enzymatic removal of its phosphate and carbohydrate groups has been shown to result in a denaturation temperature lower by about 5 °C (Kitabatake et al., 1988b; Shirai, 1997). The recombinant ovalbumin had no post-translational

modification (Fig. 8). The lower denaturation temperature of recombinant ovalbumin may be, therefore, accounted for by the absence of post-translational modifications of the recombinant proteins.

Recombinant ovalbumin also undergoes transformation into a thermostabilized form by an alkaline treatment. The use of recombinant ovalbumin would be crucial for investigating the molecular mechanism for transformation of the native form into S-ovalbumin. In regard to the formation of S-ovalbumin, two different mechanisms have been proposed so far: the first is the conformational mechanism, and the other is the covalent modification mechanism.

According to the mechanism proposed by Huntington et al. (1995a), transformation includes a large conformational transition from the native form into a thermostabilized, loop-inserted conformer. In this model, S-ovalbumin has been emphasized to be a conformer of native ovalbumin rather than a chemically distinct species. The results of our recent study, however, make it very unlikely that S-ovalbumin is a simple conformer of ovalbumin with the same covalent structure; either ovalbumin or S-ovalbumin has been found to be correctly refolded from the fully denatured state into each original non-denatured form, but not into a common stabilized form as evaluated by its thermal stability (unpublished data).

As a covalent modification mechanism, phosphoserine residues in proteins frequently undergo β-elimination, yielding dehydroalanine residues upon thermal processing and exposure to alkaline conditions; the dehydroalanine residues may further react with the ε-amino groups of the lysine residue to form lysinoalanine crosslinks, or with the thiol groups of the cysteine residues to form lanthionine crosslinks (Singh, 1991) The data in the present report, however, demonstrate that non-phosphorylated recombinant ovalbumin also underwent transformation into a thermo-stabilized form by an alkaline treatment (Figs. 8 and 6). Likewise, it has been observed that non-

phosphorylated egg white ovalbumin (A_3-ovalbumin) can be transformed into S-ovalbumin by an alkaline treatment (unpublished data). These results clearly exclude the possibility that β-elimination in the phosphoserine residues is a crucial step in the formation of S-ovalbumin.

As an alternative covalent modification, the deamidation mechanism may be a strong candidate, but it is still not proven; a differential net charge has been detected for egg white ovalbumin and S-ovalbumin (Nakamura et al., 1981) but ammonia released from the protein in an alkaline condition was much less than the stoichiometric amount (Johnson et al., 1989). The present report indicates that the site-directed mutagenesis approach to recombinant ovalbumin should be a crucial tool for elucidating the covalent modification mechanism for S-ovalbumin formation.

CHAPTER 3.

Analysis of thermostabilization accompanying cleavage at P1-P1' position in an ovalbumin variant, R339T.

A dynamic conformational change is caused in inhibitory serine protease inhibitors (serpins) by the cleavage at P1-P1' bond (Löbermann et al., 1984). The coformational change is a driving force for translocation the proteinase trapping at P1-P1' position and goes by the name of loop insertion. The inhibitory activity arises by the trapping of the covalent acyl-ester intermediate formed between the serpin and proteinase and a translocation of the proteinase from one side of the serpin to the other side (Wright & Scarscadle, 1995; Gettins et al., 1996). The hinge point for this loop insertion is at P15-P14 position, with the side chain of P14 residue becoming buried in the hydrophobic interior of serpin upon the loop insertion (Hood et al, 1994; Huntington et al., 1995b; Davis et al., 1992).

Ovalbumin is classified into a member of the serpin superfamily from a high homology in the primary structure but not shows any inhibitory activities (Hunt & Dayhoff, 1980). The tertiary structure also is similar to that of serpins, which has three β-sheets and nine α-helixes (Gettins et al., 1996). Ovalbumin is not caused to insert the reactive center loop, so the protein is noninhibitor. One of the causes is considered that the loop insertion is not occurred for the bulk and polar of the side chain in Arg339 residue corresponding to P14 site in serpin (Wright et al., 1990). The P14 residue can not bury in the hydrophobic interior for the side chain. Recently, a variant changed Arg339 residue to serine residue with a small and nonpolar side chain has been obtained in ovalbumin (Huntington et al., 1997). The variant occurs the loop insertion by cleavage at P1-P1' position (Huntington et al., 1997). The denaturation temperature of the cleaved form, however,

shows that a part of the loop is inserted. The side chain of serine residue will cause the partial insertion. In most inhibitory serpins, the P14 residue is threonine residue, which has a small uncharged side chain (Huber & Carrell, 1989). The side chain of threonine residue is a little larger than that of serine residue. A little difference will affect to the extent of loop insertion.

The disturbance of the loop insertion causes the release of the proteinase by the hydrolysis of the covalent acyl-ester inter-mediate (Engh et al., 1995). If the covalent acyl-ester bond in the intermediate is stable, serpins must show the inhibitory activity without loop insertion. In the case of a variant α1-proteinase inhibitor, which is changed threonine residue at P14 position to arginine residue, the variant has not been disturbed the reactive center loop insertion or functioning of α1-proteinase inhibitor as an inhibitor of proteinase but does significantly alter the relative rates of the substrate and inhibitory pathways in favor of the former by reducing the rate of latter reaction (Huntington et al., 1995b). In the other inhibitory serpins such as antithrombin III (Hood et al, 1994) and C1-inhibitor (Davis et al., 1992), which has been mutated P14 residue to bulky residue, the serpins have been recognized as the substrate by serine protease. The alteration at P14 position is caused by a slower rate of the loop insertion in the mutant. The ovalbumin is required the induction the loop insertion and the stability of the covalent bond to show inhibitory activity.

In this chapter, the arginine residue at P14 site in ovalbumin was changed to threonine (R339T), which is preserved in most serpins (Wright et al., 1990). Ovalbumin is cleaved at P1-P1' and P8-P7 positions by subtilisin Carlsberg (Ottesen, 1958) and at P1-P1' position by elastase (Wright, 1984). The variant after the treatment by PPE is not cleaved at P8-P7 position by subtilisin Carlsberg, which attacks at P8-P7 position following to cleavage at P1-P1' position in egg white ovalbumin (Ottesen, 1958). The variant is largely thermostabilized by the cleavage at P1-P1' position with PPE. The variant after a treatment at a low concentration of subtilisin was

contained two species, which showed the differential mobility on SDS-PAGE. This occurrence of two species can be attributed to competition between insertion of the reactive center loop into β-sheet A and the cleavage at P8-P7 by subtilisin. These results demonstrated that the reactive center loop of R339T was inserted into β-sheet A by the cleavage at P1-P1' bond. Furthermore, the rate of loop insertion due to subtilisin treatment was followed using SDS-PAGE analysis. This rate showed an independence on the concentration of subtilisin with the rate constant of 3.9×10^{-3} s^{-1}.

Material and methods

Materials.

Ovalbumin was purified from fresh egg white by crystallization three times (Sörensen et al., 1915). Trifluoroacetic acid (TFA) and dimethyl formamide (DMF) were purchased from Wako Pure Chemical Industries. Phenylmethylsulfonyl fluoride (PMSF) was from Nacali tesque. Subtilisin Carlsberg, from *Bacillus licheniformis* (protease type VIII), porcine pancreatic elastase (EC 3.4.21.11), and trypsin (type XI) were purchased from Sigma Co. N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was from Sigma Co. Restriction enzymes were obtained from TaKaRa Shuzo Co. and oligonucleotides synthesized by Amesham Pharmacia biotech. T4 polynucleotide kinase was from TaKaRa Shuzo Co.

Mutagenesis.

The expression vector pET/Ova was constructed by inserting the full-length ovalbumin cDNA digested in *Nco*I-*Bam*HI site into the pET3d vector (Takahashi et al., 1995). Mutation of arginine 339 to threonine were used two synthetic oligonucleotides, 5'-GGT ACC GAG GTG G TA

GGG TCA GC-3' (the tetrad corresponding to the change to threonine and the design of *Kpn*I site underlined) and 5'-TGC TTC ATT TCT GCA TGT GC-3'. Both the synthetic oligonucleotides were phosphorylated using T4 polynucleotide kinase. Site-directed mutagenesis was performed using ExSite™ PCR-Based Sited-Directed Mutagenesis Kit (Stratagene). The DNA sequence in the amplified vectors was confirmed from *Bam*HI site to *Stu*I site fragment containing the mutation by DNA sequencing, which was carried out with ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applide Biosystems,) and analyzed with a ABI PRISM™310 genetic analyzer (Applide Biosystems). Expression vector pET/Ova(R339T) was created by ligating the *Stu*I-*Bam*HI fragment excised from mutated vector into *Stu*I-*Bam*HI doubly digested pET/Ova. The DNA sequence in the expression vectors was confirmed in the same way. *E. coli* BL21(DE3) cells were transformed with the expression vector pET/Ova(R339T). Transformants were selected on Luria-Broth (LB) plate containing 50 μg/ml ampicillin. Expression and purification of recombinant ovalbumin were accomplished essentially as was described previously. (Takahashi et al., 1995)

Production and purification of recombinant ovalbumin.

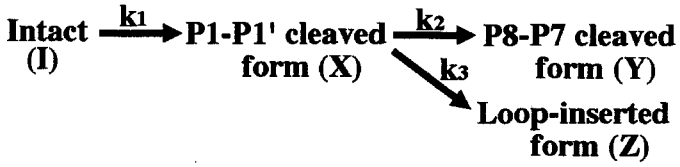
E. coli BL21 (DE3) cells harboring the expression vector, pET/Ova or pET/Ova(R339T), were incubated into 4 ml of a LB medium supplemented by 50 μg/ml of ampicillin and incubated overnight 37 °C while agitating. This culture was then transferred into two shaking flasks each containing 1 liter of the LB medium supplemented by the same concentration of ampicillin and incubated in a same way for 16 h. The *E. coli* cells collected by centrifugation (8,000 x g, 5 min) from 4-liter culture were suspended in 200 ml of a 50 mM Na-phosphate buffer containing 1 mM Na-EDTA and 1.5 mM PMSF , and disrupted with a sonicator (250 Sonifier, Branson) for 1 min 40

times at 0 °C, and centrifuged. Purification of the recombinant ovalbumin was accomplished by concentrating total proteins in the clear supernatant by precipitation in an ammonium sulfate solution (90% saturation) and subsequently dissolved in a 10 mM Na-phosphate at pH 6.0. The protein samples were dialyzed against the same buffer, applied to an anion-exchange column (HiLoad 26/10 Q-sepharose HP, Amersham Pharmacia Biotech.) and eluted by a linear 0.01 to 0.15 M gradient of the Na-phosphate buffer at pH 6.0. Ovalbumin peaks were collected. The recombinant proteins were further purified by using another anion-exchange column (Mono Q HR 10/10, Amersham Pharmacia Biotech.), the protein samples being applied to the column and eluted by the same linear gradient of the Na-phosphate buffer. The purified proteins were concentrated by the precipitation in an ammonium sulfate solution in the same way. The protein concentration was estimated from the absorption at 280 nm by using $E^{1\,\%}_{1\,cm} = 7.12$ (Glazer et al., 1963).

Rate of loop-insertion.

Ovalbumin samples (0.2 mg/ml) were incubated with the various concentration of subtilisin Carlsberg for gapped form (Ottesen, 1985) at 25 °C in 20 mM Na-phosphate buffer at pH 7.0. At various incubation times, 2.5 μl of 5 % TFA were added to 25 μl of the reaction samples. These samples were added 10 μl of SDS buffer (0.25 M Tris-HCl, pH 7.0, 4 % SDS, 40 % glycerol, 80 mM 2-mercaptoethanol) were pretreated in a boiling water bath for 2 min and were added 2.5 μl of 1.0 M Tris-base. The sample mixtures (2.5 μg of protein) were subjected to a SDS-PAGE using 10 % polyacrylamide gel according to the standard method of Laemmli, and the polypeptides on the gel were visualized with Coomassie Brilliant Blue R-250 and quantified using a flatbed scanner (GT-9000, Epson) and the software NIH image (version 1.60). The intact molecule is cleaved at P1-P1' position. In the next step, two pathways are considered by the competition of cleavage at

P8-P7 position with the loop insertion. One pathway is a pathway in which P8-P7 cleaved form appears by cleavage of P1-P1' cleaved form at P8-P7 position. Another pathway is a pathway that P1-P1' cleaved form is not cleaved at P8-P7 position by the occurrence of the loop inserted form. All steps are showed in an irreversible manner:



The quantum of each molecule at a time, t, is drawn as the following equations (1) - (3):

$$I(t)=[I]_0\exp(-k_1t) \quad (1)$$

$$X(t)+Z(t)=[I]_0\left[\frac{k_3}{k_2+k_3}+\frac{k_1-k_3}{k_2+k_3-k_1}\exp(-k_1t) - \frac{k_1k_2}{(k_2+k_3)(k_2+k_3-k_1)}\exp\{-(k_2+k_3)t\}\right] \quad (2)$$

$$Y(t)=[I]_0\left[\frac{k_2}{k_2+k_3}-\frac{k_2}{k_2+k_3-k_1}\exp(-k_1t) + \frac{k_1k_2}{(k_2+k_3)(k_2+k_3-k_1)}\exp\{-(k_2+k_3)t\}\right] \quad (3)$$

Where I, X, Y, and Z represent the quantum of intact, P1-P1' cleaved, P8-P7 cleaved, and loop inserted molecule, respectively. When the loop is not inserted, the equation (2) and (3) is changed into the equation drawn as following (4) and (5), respectively, by substitution of zero for the rate constant, k3, for loop insertion.

$$X(t)=\frac{k_1[I]_0}{k_2-k_1}(\exp(-k_1t)-\exp(-k_2t)) \quad (4)$$

$$Y(t)=[I]_0\left(1+\frac{1}{k_2-k_1}\right)(k_2\exp(-k_1)-k_1\exp(-k_2t)) \quad (5)$$

Preparation of nicked form ovalbumin by PPE.

Ovalbumin samples (1 mg/ml) were treated with 1 μg/ml of PPE in a 20 mM Na-phosphate buffer at pH 7.0 at 25 °C for 4 h. The reactions were stopped by adding 0.015 volume of 0.1 M PMSF and the mixtures were diluted with 3 volumes of 5 mM Na-phosphate buffer at pH 6.0.

The protein samples were applied to an anion-exchange column (Mono Q 5/5, Amersham Pharmacia Biotech.) and eluted in the same gradient used for initial purification of recombinant ovalbumin. Nicked ovalbumin peaks were collected.

DSC.

The thermostability of the recombinant ovalbumin samples was analyzed with a DSC (micro Cal). The protein concentration was 0.3 mg/ml in the 50 mM Na-phosphate buffer at pH 6.0. The temperature was scanned at 1 $K \cdot min^{-1}$.

CD-spectrum.

The far-UV CD spectrum was recorded at 0.2 mg/ml of protein as described by Takahashi et al. (1996). The fluorescence spectrum of ovalbumin was measured with a fluorescence spectrophotometer (model F-3000, Hitachi).

Subtilisin activities.

Spectrophotometric assays for subtilisin Carlsberg were conducted at 25 °C using 0.25 mM N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Delmar et. al., 1979) in a 20 mM Na-phosphate buffer at pH 7.0 containing 1 % DMF; the final volume was 2 ml, and the enzyme concentration ranged from 0.5 to 2.0 nM. In the Spectrophotometric assays the absorbance of the p-nitroanilide released was monitored at 410 nm with a Shimazu UV-3000 spectrophotometer. The enzyme shows a half activity in 20 mMNaPi buffer, pH 7.0 at an incubation time of 240 min.

SDS-PAGE

SDS-PAGE was carried out with 10% polyacrylamide gel at a constant current of 12.5 mA for 2.5 h according to the standard method by Laemmli (1970). Proteins were stained with 0.25% Coomassie Brilliant Blue R-250.

Results

Proteolysis of ovalbumin by elastase and subtilisin Carlsberg

Egg white ovalbumin is cleaved at P1-P1' position by PPE (Wright, 1984), and removed between P1-P1' and P8-P7 positions by subtilisin Carlsberg (Ottesen, 1958). To investigate pattern in cleavage of recombinant ovalbumin, recombinant proteins were treated by elastase and subtilisin Carlsberg and the proteolytic pattern were represented on SDS-PAGE (Fig. 10). The treatments of wild-type and variant by PPE were caused the same occurrence of a single band as egg white protein (lanes 5 and 8). This result shows both recombinant proteins were cleavage at P1-P1' position by elastase. The treatment of wild-type by subtilisin Carlsberg showed a single band with a little larger mobility than by PPE (lane 6). This result indicates that wild-type is cleaved at P8-P7 position. While the treatment of variant by subtilisin Carlsberg showed double bands with differential mobility. One band has the same mobility with the treatment by PPE. Another one has a little larger mobility than the treatment by PPE. This result shows the existence of a molecule, which can not be cleavage at P8-P7 position.

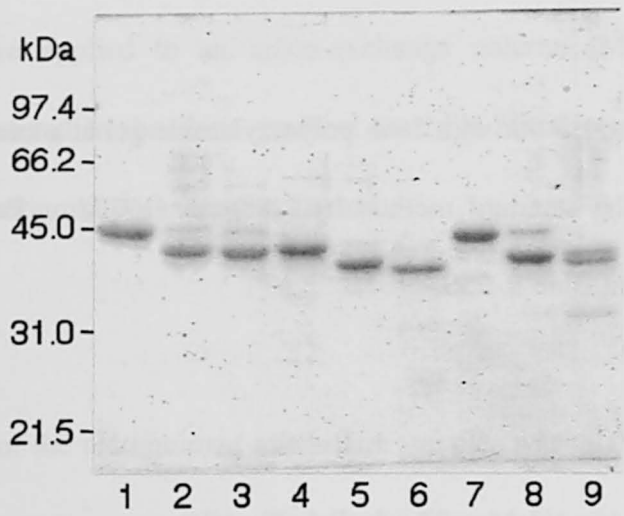


Fig. 10 Proteolysis of Ovalbumin by PPE and Subtilisin Carlsberg. Egg white ovalbumin (lanes 1, 2, and 3), wild type (lanes 4, 5, and 6), and variant (lanes 7, 8, and 9) were electrophoresed in SDS-polyacrylamide gel (10% polyacrylamide). The incubation was carried out at 25 ng/ml of PPE (lanes 2, 5, and 8) or 5 ng/ml of subtilisin Carlsberg (lanes 3, 6, 9) at 25 °C for 20 hours. The protein (2.0 μg) were subjected to the SDS-PAGE and stained with Coomassie Brilliant Blue R-250 as described in the text.

Analysis of loop insertion

To investigate whether the loop-insertion is caused by cleavage at P1-P1' position, variant cleaved at P1-P1' by elastase is purified with an anion-exchange column chromatography. The far-UV CD spectrum showed that the P1-P1' cleaved form in the variant displayed almost the same far-UV CD spectrum as that of the form in wild-type (Fig. 11).

The thermostability of recombinant proteins was evaluated by DSC. The cleaved wild-type displayed a denaturation temperature lower by 1.0 °C upon intact one (Fig.12, profiles a and b). On the other hand, the cleaved variant displayed a denaturation temperature higher by 15.8 °C (Fig. 12, profiles C and D). These results indicate the possibility that the loop is inserted into β-sheet A by the cleavage at P1-P1' position in variant.

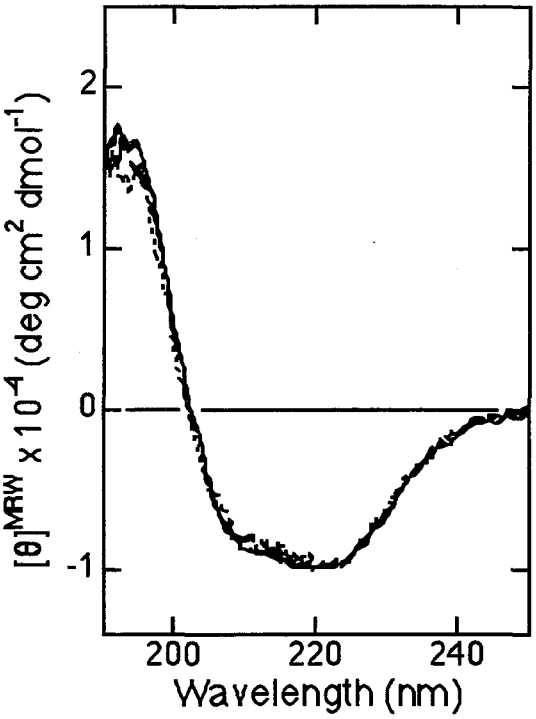


Fig. 11 Far-UV CD Spectra of Intact and Cleaved Ovalbumin. CD spectra of intact wild-type (dotted line), cleaved wild-type (long dashed line), intact variant (dashed line), and cleaved variant (solid line) were measured in the far-UV region (190-250 nm). The cleaved forms were prepared by the incubation of ovalbumin with 0.1 volume (w/w) of PPE at 25 °C and the purification with an anion-exchange column as described in text.

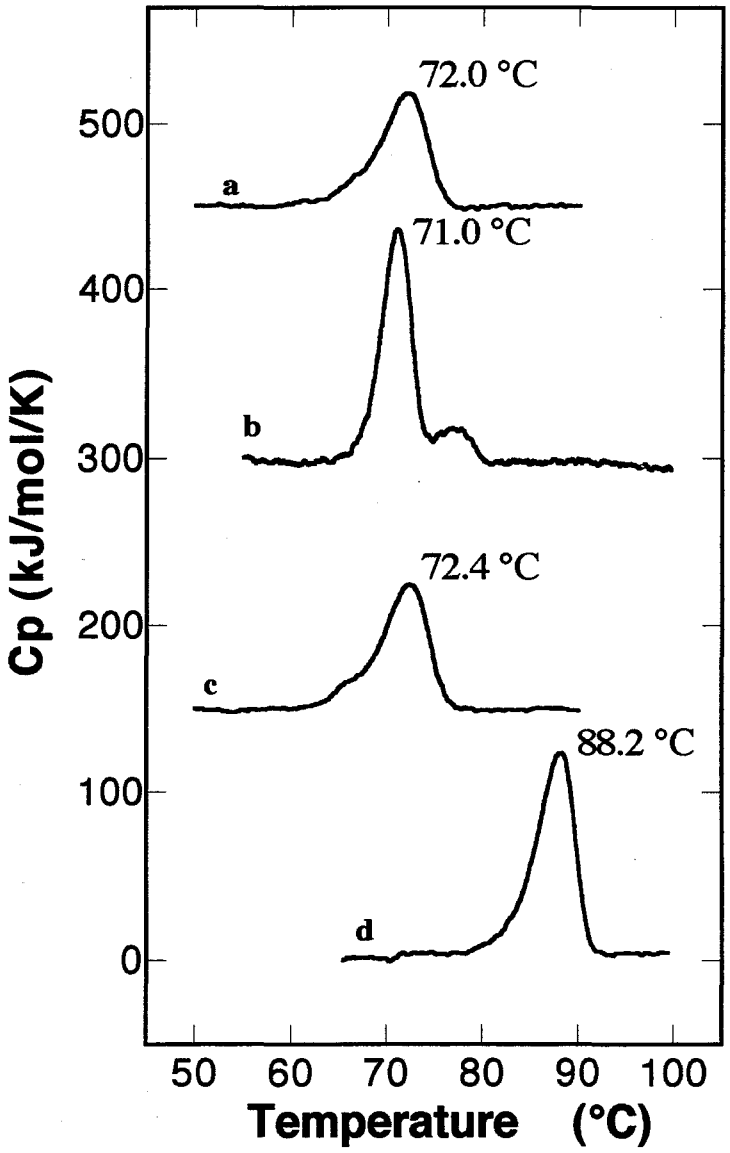


Fig. 12 Thermostability of Cleaved ovalbumin by PPE. The thermostability of intact wild-type (a), cleaved wild-type (b), intact variant (c), and cleaved variant (d) were analyzed with a DSC. The temperature was scanned at 1 $K \cdot min^{-1}$ and the protein concentration was 0.3 mg/ml in a 10 mM Na-phosphate buffer at pH 6.0. Endothermic transition profiles have been arbitrarily shifted on the ordinate scale for clarity.

To confirm whether the loop insertion is caused in the cleaved form of variant or not, the treatment of the cleaved form of variant by subtilisin was carried out (Fig. 4). Ovalbumin is cleaved at P1-P1' and P8-P7 position by subtilisin (Ottesen, 1958). If the loop of P1-P1' cleaved form is not inserted into b-sheet A, the protein will be cleaved at P8-P7 position on the loop. As shown in Fig. 13, the P1-P1' cleaved form is cleaved at P8-P7 position by subtilisin Carlsberg in wild-type but not cleaved in variant. This result strongly suggests that the loop insertion is carried out in variant. Furthermore, I tried to determine the rate of the loop insertion in the variant.

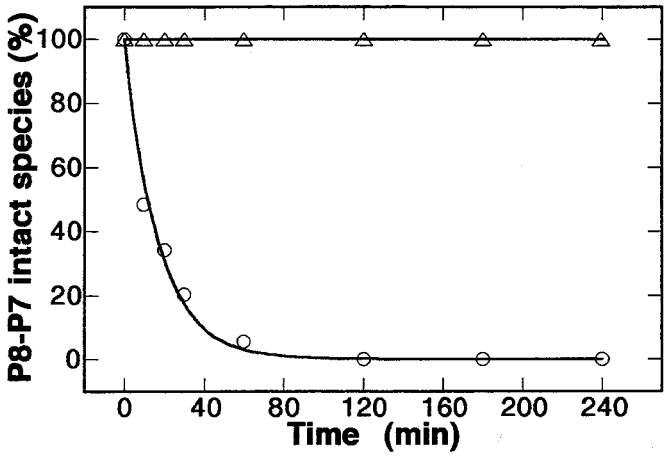


Fig. 13 Susceptibility of Ovalbumin Cleaved at P1-P1' Position by PPE to Subtilisin. Wild-type (circle) and variant (triangle) cleaved at P1-P1' was digested with subtilisin (20 ng/ml) in 20 mM Na-phosphate buffer, pH 6.0 at 25 °C for various times, and the analyzed for SDS-PAGE as described in the text. The band intensity was measured with a flatbed scanner and a software. The data for wild-type is fitted by equations (7).

Kinetic studies of loop insertion.

As shown in Fig. 10, the variant cleaved by subtilisin Carlsberg showed double bands with differential mobility. The result shows that the cleavage at P8-P7 position compete with the loop insertion. The kinetics of the loop insertion was studied by tracing a transition of the population of the triplet bands with differential mobility on SDS-PAGE (Fig. 14).

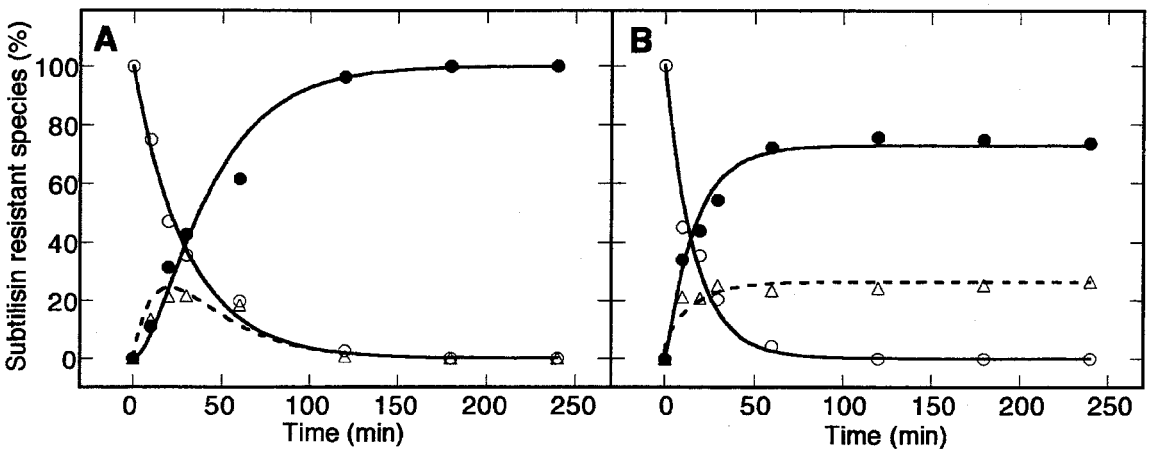


Fig. 14 Kinetics of Loop insertion probed by the Susceptibility of the Variant to Proteolysis at P8-P7 Position by Subtilisin. Ovalbumin (0.2 mg/ml) is incubated with 20 ng/ml of subtilisin Carlsberg in 20 mM Na-phosphate buffer, pH 6.0 at 25 °C for various times. The reactions were stopped by adding 0.1 volume (v/v) of 5 % TFA. The proteins (2.5 μg) were subjected to SDS-PAGE and stained with Coomassie Briliiant Blue R-250 as described in the text. The band intensities of intact (open circle) molecule, the sum of P1-P1' cleaved and loop inserted molecule (triangle), and P8-P7 cleaved molecule (closed circle) is quantified by a flatbed scanner and a software. In panel A, the data for wild-type is fitted by equations (1), (4), and (5). In panel B, the data for the variant is fitted by equations (1), (2), and (3).

The data is fitted by equations (1), (4), and (5) in panel A and equations (1), (2), and (3) in panel B. The rate constant, k_1, for the cleavage at P1-P1' position was estimated to be 5.4×10^{-4} sec^{-1} (correlation coefficient, R=0.997) in the wild-type and 1.0×10^{-3} sec^{-1} (R=0.993) in variant,

respectively. The rate constant, k_2, for the cleavage at P8-P7 position was estimated to be 1.1 x 10^{-3} sec^{-1} (R=0.995) in the wild-type and 1.1 x 10^{-2} sec^{-1} (R=0.996) in variant, respectively. The rate constant, k_3, for the loop insertion in variant was estimated to be 4.5 x 10^{-3} sec^{-1} (R=0.992). As shown in Fig. 15, the rate constants, k_1 (panel A) and k_2 (panel B), were depend on the concentration of subtilisin Carlsberg.

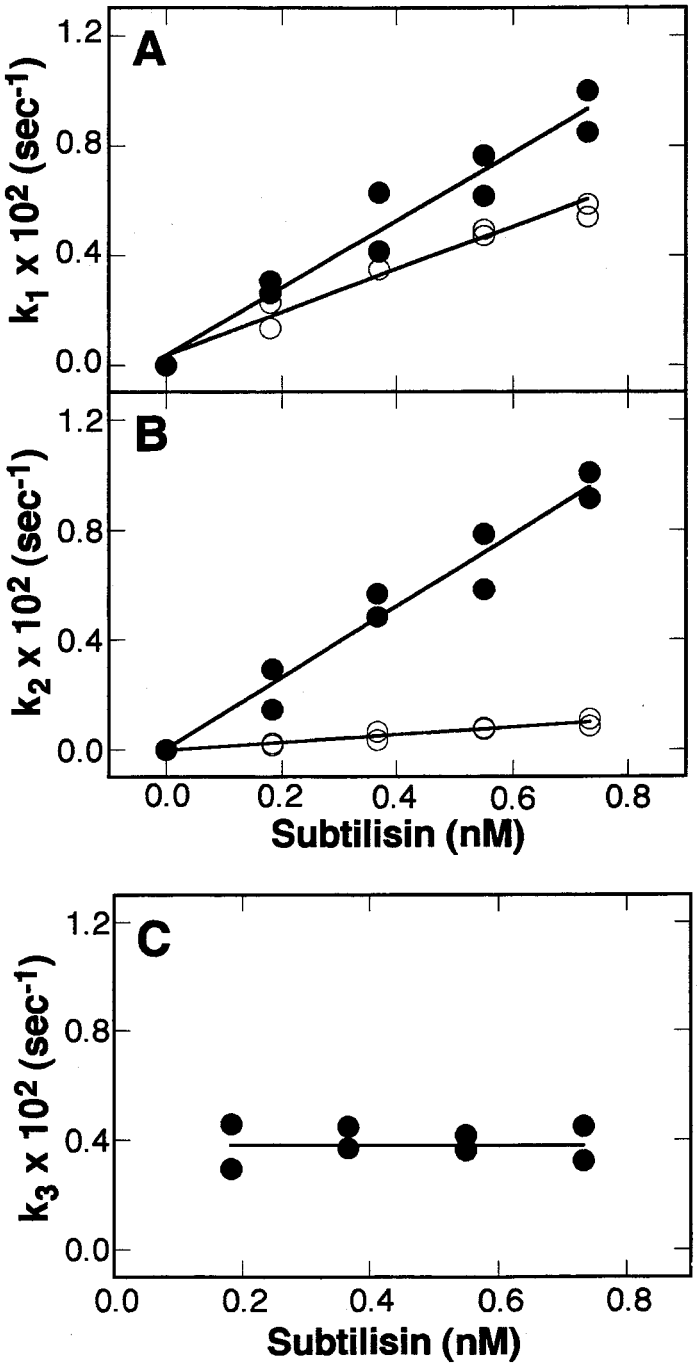


Fig. 15 Dependence of the Rate Constant on the Concentration of Subtilisin. Wild-type (open circle) and the variant (closed circle) at 0.2 mg/ml were incubated at various concentration of subtilisin Carlsberg in 20 mM Na-phosphate buffer, pH 6.0 at 25 °C for various times. The rate constants, k_1 (panel A), k_2 (panel B), and k_3 (panel C), were estimated by fitting the data to the equations (1)-(5).

This result shows that k_1 and k_2 are second order rate constants. The second order rate constants for the cleavage at P1-P1' were determined from the slopes to be 1.2 x 10^6 $M^{-1}sec^{-1}$ and 7.8 x 10^5 $M^{-1}sec^{-1}$ in the variant and in wild-type, respectively. The second order rate constants for the cleavage at P8-P7 were determined from the slopes to be and 1.3 x 10^7 $M^{-1}sec^{-1}$ and 1.4 x 10^6 $M^{-1}sec^{-1}$ in the variant and in the wild-type, respectively. The variant showed about 2 times rate constant for the cleavage at P1-P1' position and 10 times rate constant for the cleavage at P8-P7 position by the replacement of the P14 residue. While the rate constant, k_3 (Fig. 15, panel C), is independent on the concentration of the enzyme and the first order rate constant determined from the intercept in panel C of Fig. 15 was 3.9 x 10^{-3} sec^{-1}. This result shows that the rate constant is the first order rate constant. To investigate whether these experiments are carried out in the extent of the pseudo-first order reaction, the rate constant, k_1, k_2, and k_3 were estimated by the same way at various concentrations of ovalbumin (Fig. 16). This result shows that these experiments were carried out in the correct conditions.

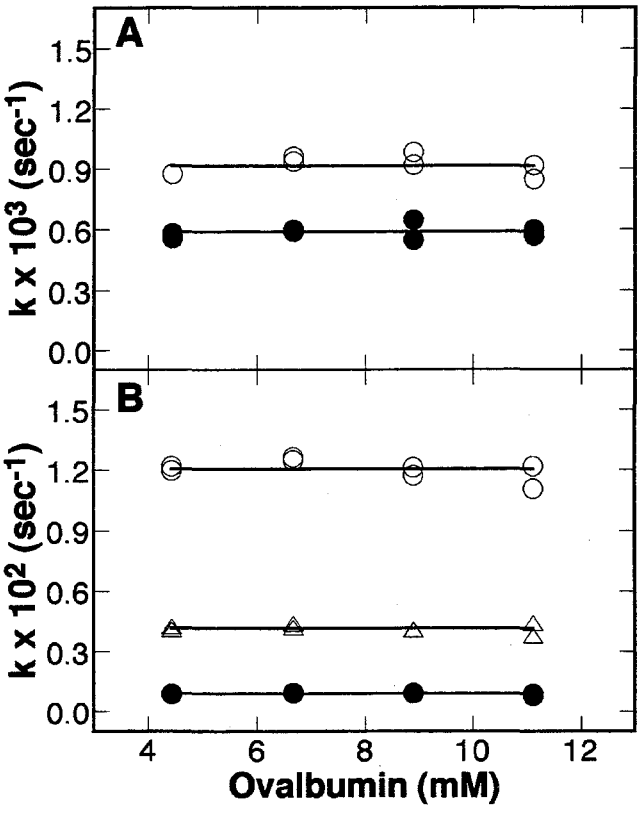


Fig. 16 Dependence of the Rate Constant on the Concentration of Ovalbumin. Wild-type (panel A) and the variant (panel B) at various concentrations were incubated with 20 ng/ml of subtilisin Carlsberg in 20 mM Na-phosphate buffer, pH 6.0 at 25 °C for various times. The rate constants, k_1 (closed circle), k_2 (open circle), and k_3 (triangle), were estimated by fitting the data to the equations (1)-(5).

Discussion

Ovalbumin is a noninhibitory member of the serpin family (Hunt & Dayhoff, 1980). The protein is noninhibitor for one reason that the bulk and polar side chain of Arg339 residue hinders the loop insertion which is characteristic in serpin (Wright et al., 1990). This dynamic conformational change is caused in inhibitory serpins by the cleavage at P1-P1' bond (Löbermann et al., 1984). The inhibitory activity arises by the trapping of the covalent acyl-ester intermediate formed between the serpin and proteinase and a translocation of the proteinase from one side of the serpin to the other side (Wright & Scarscadle, 1995; Gettins et al., 1996). The driving force for the translocation is an insertion of reactive center loop into β-sheet A. The hinge point for this loop insertion is at P15-P14, with the side chain of P14 residue becoming buried in the hydrophobic interior of serpin upon the loop insertion (Hood et al, 1994; Huntington et al., 1995b; Davis et al., 1992). In this chapter, the arginine residue at P14 position in ovalbumin was changed to threonine (R339T), which is preserved in most serpins. The thermostability of the variant cleaved at P1-P1' position by elastase increased by 15.8 °C (Fig. 12). In the case of a variant that is changed the P14 to serine residue, the variant ovalbumin resulted in an 11 °C increase in the themostability by the cleavage at P1-P1' position (Huntington et al., 1997). The replacement of the P14 residue to threonine residue is caused to transform into more stable form than the replacement to serine residue. This difference of thermostability results from the difference in the bulk of the side chain between threonine and serine residues. The side chain of threonine residue is lager than that of serine residue, so the side chain may easily make the loop to insert by spreading the space between strand 3A and strand 5A and the bulk may be suitable to fix the loop inserted. The increase of thermostability in R339T is almost the same value as an increase showed in an ovalbumin variant, which is replaced the P14 arginine residue by serine

residue in combination with changes of P12-P10 to alanine residue, cleaved at P1-P1' position (Huntington et al., 1997). The P12 and P11 residues in ovalbumin are valine, which has a larger side chain than serine residue. The quartet variant may be unnecessary to spread the space between the strands for the reason of changes to the smaller side chain.

The variant R339T does not show any inhibitory activities to subtilisin (unpublished data). There is a possible explanation to account for the absence of inhibitory properties in the variant, despite the occurrence of the loop insertion. The explanation is that the rate of loop insertion is still not high enough. The rate must be larger than the rate of hydrolysis of the covalent acyl-ester bond that trapped the enzyme by attachment to the carboxyl of P1 residue. In the case of plasminogen activator inhibitor type 1 which is an inhibitory serpin, the rate constant for the loop insertion has been showed to be 3.4 sec^{-1} by recent fluorescence resonance energy transfer measurements (Shore et al., 1995). The inhibitory serpin has about 1000 times rate constant for loop insertion in comparison with that of the ovalbumin variant (Fig. 15). For ovalbumin show any inhibitory activities, it will be essential that loop insertion is speeded up. Although the composition of amino acid residues on the loop has attracted a great deal of attention, it will be important to investigate the role of strand 3A and 5A as a saucer.

The variant showed about 2 times rate constant for the cleavage at P1-P1' position and 10 times rate constant for the cleavage at P8-P7 position by the replacement of the P14 residue (Fig. 15). The P1-P1' and P8-P7 positions will be easy to be attacked by exposure to solvent in the variant. The increase of accessibility to the P8-P7 position may be caused by that the loop is largely moved accompanying to insert the loop. The cleavage of P1-P1' cleaved form at P8-P7 was represented by an irreversible scheme:

$$\underset{\text{form (X)}}{\text{P1-P1' cleaved}} \xrightarrow{k_4} \underset{\text{form (Y)}}{\text{P8-P7 cleaved}}$$

The first order rate constant of cleavage at P8-P7 by subtilisin was estimated to be 9.5×10^{-4} sec^{-1} from Fig. 13 by fitting of equation (7):

$$X(t)=[X]_0 \exp(-k_4 t) \qquad (7)$$

The second order rate constant was estimated to be 1.3×10^6 $M^{-1}sec^{-1}$ by the concentration of subtilisin into the first order rate constant for cleavage of the cleavage at P8-P7 position. The second rate constant estimated from Fig. 13 was consistent with that estimated from Fig. 15. The P1-P1' cleaved form was prepared by a purification with anion exchange column and concentration with ammonium sulfate. This consistency shows that loop insertion does not occur in the cleaved form of wild-type during the storage.

Ovalbumin is transformed into a thermostable form, S-ovalbumin in the stored egg shells. As described in CHAPTER 2, the recombinant ovalbumin was also transformed into thermo-stabilized form by the alkaline treatment. Huntington et al. (1995a) demonstrated a relationship between formation of S-ovalbumin and loop insertion. This transformation mechanism, however, can not be explained by loop insertion alone.

SUMMARY

Ovalbumin shows three unique characteristics in the relation to the protein folding, transport and functional expression. Firstly, ovalbumin is secreted without any cleavages of the signal sequence for the secretion. Secondly, ovalbumin is a transformed into a more heat stable form, S-ovalbumin, in stored shell eggs and on process of embryogeny. Lastly, ovalbumin is a member of serine proteinase inhibitor (serpin) superfamily but does not have any inhibitory activities. The author tried to approach to these characteristics utilizing the ovalbumin expression system.

In chapter 1, the author indicated that the recombinant ovalbumin was secreted into periplasmic space of *E. coli* cells without any cleavage of N-terminal region. The recombinant protein in the periplasmic space did not contain the disulfide bond, Cys73-Cys120. These results suggest that the recombinant protein was secreted on a different pathway from the ordinary pathway utilized in secretory proteins.

In chapter 2, the recombinant ovalbumin was purified from the cytoplasm of *E. coli*, and its structural characteristics are compared with those of egg white ovalbumin. The peptidic structure and conformation of the recombinant protein were essentially the same as those of egg white protein. However, none of the post-translational modifications known in egg white ovalbumin, including N-terminal acetylation, phosphorylation, and glycosylation, was detected in the recombinant protein. Further, the recombinant protein was transformed into a more thermostable form by an alkaline treatment. The author presented that the post-translational modifications were not related to the transformation into S-ovalbumin.

In chapter 3, the author prepared an ovalbumin variant, R339T, which was changed Arg339 residue to Thr residue by site-directed mutagenesis. The variant was transformed by the cleavage

at the P1-P1' position into a protein form that displayed thermostability higher by 15.8°C. The increase of thermostability strongly suggests that the loop insertion was caused by the cleavage at the P1-P1' position in the variant. Furthermore, the author established the method in kinetics of loop insertion by proteolytic cleavage analysis. The rate for loop insertion, k_3, was estimated to be 3.9×10^{-3} sec^{-1}. The rate for the cleavage at P1-P1' position, k_1, was 1.2×10^6 $M^{-1}sec^{-1}$ and 7.8×10^5 $M^{-1}sec^{-1}$ in variant and wild type, respectively. And the rate for the cleavage at P8-P7 position, k_2, was 1.3×10^7 $M^{-1}sec^{-1}$ and 1.4×10^6 $M^{-1}sec^{-1}$ in variant and wild type, respectively.

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LIST OF PUBLICATIONS

1. Yasuhiro Arii, Nobuyuki Takahashi, Eizo Tatumi, and Masaaki Hirose: Structural properties of recombinant ovalbumin and its transformation into a thermostablized form by alkaline treatment, *Biosci. Biotechnol. Biochem.*, **63**, 1392-1399 (1999).

2. Yasuhiro Arii, Nobuyuki Takahashi, Eizo Tatsumi, and Masaaki Hirose: Periplasmic secretion in Escherichia coli of chicken ovalbumin without signal cleavage and disulfide formation, Maniscript in preparation.

3. Yasuhiro Arii and Masaaki Hirose: Loop insertion Mechanism in an ovalbumin variant R339T as probed by proteolytic cleavage kinetics. (Kinetics of the loop insertion in an ovalbumin variant R339T by proteolytic cleavage analysis), Maniscript in preparation.