

Some structural properties of ovalbumin heated at 80°C in the dry state

Naotoshi Matsudomi *, Hidekazu Takahashi, Takeshi Miyata

Department of Biological Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan

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Abstract

Dry heating of ovalbumin at 80°C improved the gel properties, including the gel strengthening and transparency upon the subsequent heating for gelation [Matsudomi, N., Ishimura, Y., & Kato, A., (1991). Improvement of gelling properties of ovalbumin by heating in dry state. *Agricultural and Biological Chemistry*, 55, 879–881.]. Some structural properties of the dry-heated ovalbumin responsible for such gel properties were studied. The electrophoretic patterns revealed the formation of ovalbumin polymer through hydrophobic and disulfide protein-protein interaction during heating in the dry state. The slight changes in circular dichroism spectrum and surface hydrophobicity indicated mild conformational changes in ovalbumin molecules. Differential scanning calorimetry thermograms showed that enthalpy of denaturation of ovalbumin was markedly decreased with an increase of dry-heating time. The deamidation of ovalbumin was found to occur during the dry-heating process, and the degree of deamidation reached 10% after dry heating at 80°C for 7 days. It was assumed that the partially unfolded and deamidated ovalbumin produced by the dry heating forms specific soluble aggregates during subsequent heating for gelation, resulting in the formation of an ordered gel matrix. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Ovalbumin; Dry heating; Deamidation

1. Introduction

Dry heating is one of the most promising approaches to improve the gelling properties of dried egg white protein powder (Kato, Ibrahima, Watanabe, Honma, & Kobayashi, 1989). The effects of heating egg white protein in a dry state at 80°C on their gelling properties were characterized with the relation to their protein denaturation (Kato, Ibrahima, Watanabe, Honma, & Kobayashi, 1990; Kato, Ibrahima, Takagi, & Kobayashi, 1990; Mine, 1996, 1997). However, the mechanisms responsible for the significant improvement of gelling properties of the dry-heated egg white protein are not fully explained because of the complicated interactions among the different proteins, in addition to the denaturation of egg white occurring in dry heating. On the other hand, we have shown improvements in the gel properties of ovalbumin (OVA), the main constituent of egg white protein, by dry heating at 80°C, the dry-

heated OVA forming clearer and firmer gels when compared to non-heated OVA (Matsudomi, Ishimura, & Kato, 1991). Therefore, monitoring the changes in protein structure of ovalbumin upon dry heating, differing from egg white protein in a complex system, will help to further the understanding of the relationship between the structure and gelling properties of the dry-heated egg white protein. In addition, we showed that the dry heating of OVA lowered the sensitivity to NaCl and the degree of aggregation of soluble aggregates when its OVA was heated in solution (Matsudomi, Yamamoto, Fujimoto, & Kobayashi, 1993). Such properties of the soluble aggregates might be due to a substantial protein denaturation occurring in dry heating. However, it is unclear how the molecular properties of OVA might be affected during heating in the dry state. The molecular basis of structural changes of dry-heated OVA is important for an understanding of the effects of dry heating on gelling properties. This study was conducted to characterize denaturation of OVA occurring during dry heating, in order to clarify the gelling properties, including the strengthening and the transparency of heat-induced OVA gels.

* Corresponding author. Tel.: +81-839-33-5853; fax: +81-83-933-5820.

E-mail address: naotoshi@agr.yamaguchi-u.ac.jp (N. Matsudomi).

2. Materials and methods

2.1. Materials

OVA was isolated from fresh hen egg white by a crystallization method in sodium sulfate according to the method of Kekwich and Cannan (1936), and then freeze-dried after adjusting pH to 7.0. *cis*-Parinaric acid was from Sigma Chemical Co. Protein solution was prepared with 10 mM sodium phosphate buffer (pH 7.0) unless otherwise mentioned. All chemicals used in this study were of analytical grade.

2.2. Heat treatment of OVA

Dry heating was conducted on the freeze-dried OVA (moisture content: 5.7%) for several days (0–10 days) at 80°C, as described previously (Matsudomi et al., 1991). All dry-heated OVA samples were kept in a desiccator at 4°C pending analysis.

2.3. Measurement of solubility

Solubility was measured by determining the protein concentration in the dry heated OVA sample (1%) in 10 mM phosphate buffer (pH 7.0) followed by centrifugation (5200×*g*) for 10 min. The serum was filtered by a membrane filter (0.45 µm). Protein concentration was determined according to the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951). Protein solubility of dry heated sample was represented as the ratio to that of non-heated sample.

2.4. Gel filtration

Gel filtration was carried out by high-performance liquid chromatography on a TSK gel G3000SW column (Tosoh; 7.5×300 mm) equilibrated with 100 mM phosphate buffer (pH 7.0). The 20 µl of 0.1% protein solution was applied on the column at a flow rate of 0.5 ml/min. A variable wavelength UV monitor (Hitachi, 655A) was used to monitor the effluent at 280 nm.

2.5. Gel electrophoresis

Dry-heated OVA samples were resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie blue R, according to the method of Laemmli (1970). Electrophoresis was done in both reducing and non-reducing conditions in the presence and absence of 2-mercaptoethanol (2-ME), respectively. In order to assess the structural changes of dry-heated OVA, native-PAGE without SDS and reducing agent was also performed in 7.5% polyacrylamide gel sheet, as described by Davis (1964).

2.6. Measurement of surface hydrophobicity

Surface hydrophobicity was measured by the fluorometric method using *cis*-parinaric acid as described by Kato and Nakai (1980). Ethanolic solution of *cis*-parinaric acid (3.6×10^{-3} M) was prepared and equimolar butylated hydroxytoluene was added as an antioxidant. Ten µl of the *cis*-parinaric acid solution was added to 2 ml of protein solutions (0.1, 0.05, 0.025, and 0.0125%). The mixtures were excited at 325 nm, and the relative fluorescence intensities were measured at 420 nm in a spectrophotofluorometer (Aminco-Bowman, J-8962). The reading of relative fluorescence intensity was adjusted to 1.0 when 10 µl of *cis*-parinaric acid solution was added to 2 ml of 10 mM phosphate buffer (pH 7.0), in the absence of protein. The initial slope (S0), fluorescence intensity/% protein, was calculated from the fluorescence intensity vs. protein concentration plot.

2.7. Circular dichroism (CD) spectra

CD spectra were taken using a spectropolarimeter (Jasco, J-600) in the far-ultraviolet region (200–260 nm) with 0.005% protein solution in 1.0 cm path length cuvette. The temperature of the cuvette was maintained at 25°C with a circulating water bath. The accuracy of the data was improved by averaging three scans integrated using a software program (DP-J600/PC System, Ver. 1.39, Copyright by Jasco). CD spectra were represented in terms of mean residue ellipticity (degree cm²/dmol).

2.8. Differential scanning calorimetry (DSC)

The thermal characteristics were examined by a thermal analyzer (Seiko Instrument, DSC-120). Protein solution (1%, 40 µl) was sealed in a silver pan. The pan was heated in the calorimeter at a linear rate of 2°C/min over the range 25–100°C. The denaturation temperature (Td) and enthalpy of denaturation (ΔH) were computed from the thermogram by using a software program (SSC5200H Disk Station, Ver. 2, Copyright by Seiko Instrument).

2.9. Ion-exchange chromatography

Protein solution (0.2%, 5 ml) was applied to a DEAE-Toyopearl 650 M column (Tosoh, 18×200 mm) equilibrated with 10 mM phosphate buffer (pH 7.0) and eluted with a linear gradient of 0–0.5 M NaCl in the same buffer. The 3-ml fractions were collected and measured at 280 nm.

2.10. Measurement of sulfhydryl thiol (SH) groups

Free SH groups were determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) according to the

procedure of Ellman (1959). To 1 ml of 0.1% protein solution was added 1 ml of 0.1 M Tris–glycine buffer (pH 8.0) containing 0.01 M EDTA (for surface SH groups) and the same buffer containing 5% SDS (for total SH groups). After incubation at 40°C for 30 min, 50 μ l of DTNB solution (4 mg in 1 ml of 0.1 M Tris–glycine buffer, pH 8.0) was added and then incubated at 25°C for 10 min. The color was read at 412 nm. The molar extinction coefficient of 13600 M⁻¹ cm⁻¹ at SH groups in the OVA molecule modified by DTNB was used. The SH content was calculated by moles of SH per mole of OVA.

2.11. Measurement of deamidation of protein

Protein deamidation was analyzed by measuring the ammonia generated during the reaction. The samples were dialyzed overnight against deionized water to remove the free acids and freeze-dried. Then, the sample (100 mg) was dissolved in 2.0 ml of 2 N HCl and refluxed for 3 h. An equal volume of 10% trichloroacetic acid solution was added to the sample to precipitate the protein and centrifuged. Ammonia in the supernatant was analyzed using an ammonia electrode (Orion Research Inc.). The degree of deamidation of the samples was determined from the values of ammonia as follows:

Deamidation percentage (%) = (value of native protein – value of dry-heated protein / value of native protein) $\times$ 100

3. Results and discussion

The effect of heating at 80°C in the dry state on the solubility of OVA is shown in Fig. 1. The solubility at pH 7.0 of the dry-heated OVA samples was high and remained relatively constant within 7 days. However, a slight loss (about 5%) of water solubility of the protein was observed in the sample dry-heated for 10 days. Any browning of each OVA sample was not observed within heating for 10 days. In order to obtain information regarding the molecular size of dry-heated OVA samples, gel filtration and electrophoresis were carried out.

Fig. 2 shows the gel filtration patterns of OVA and the dry-heated samples. The elution pattern of non-heated OVA indicated a single peak with a retention time of 19.5 min. On the other hand, the sample dry-heated for 1 day gave three peaks. These peaks seem to correspond to the monomer, dimer and trimer of OVA molecule, when estimated from the retention time of the standard proteins. The patterns of dry-heated OVA samples showed that polymerization proceeds during the dry heating at 80°C. The polymer components having a apparent molecular weights ranging more than 669 kDa (molecular marker: thyroglobulin) were

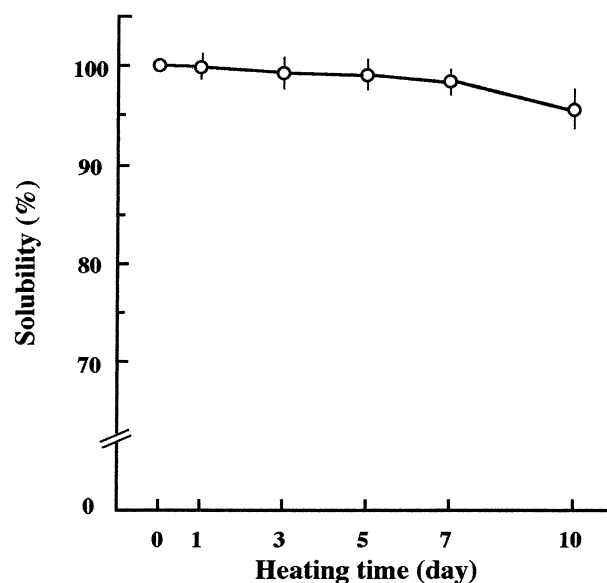


Fig. 1. Changes in the solubility of dry-heated ovalbumin at 80°C for various periods of time. Data are the average of triplicate measurements.

detected in the samples dry-heated for more than 5 days, although the monomer of OVA in these samples also remained as the main component. Interestingly, the monomeric fraction in the dry-heated samples was found to be eluted at only slower retention time than non-heated OVA.

Gel electrophoreses were performed to elucidate the binding type of the polymer formed by the dry heating (Fig. 3). Native-PAGE without SDS and reducing agent is presented in Fig. 3A. Non-heated OVA was dissociated into three bands depending on the number of phosphoserine residues. The patterns of dry-heated samples revealed the formation of aggregates that could migrate slowly into the gel, and the intensities of the bands of aggregates increased with increased heating time in the dry state. It was confirmed that polymerization of OVA molecules occurred during the dry heating at 80°C, as shown in the gel filtration patterns (Fig. 2). Then, in order to assess the binding type of aggregates, SDS-PAGE in the presence (Fig. 3C) and absence (Fig. 3B) of 2-ME, respectively, was performed. It can be clearly seen from Fig. 3B that the dry heating induced substantial aggregation in the protein leading to coexistence of different population of monomeric and multimeric OVAs. When OVA was dry-heated over 3 days, OVA aggregates was observed, and the intensities of the bands of aggregates stepwise increased, the relative concentrations of OVA monomer decreasing with the increased heating time. In the presence of SDS and 2-ME (Fig. 3C), the dimer and oligomer of OVA were considerably reduced to the monomers. However, most aggregates having higher molecular weights remained undissociated. These results indicated that disulfide

bond formation was not the major mechanism involved in the formation of aggregates, and that binding forces among aggregates in dry-heated OVA samples would be tight. It is interesting to note that some bands having

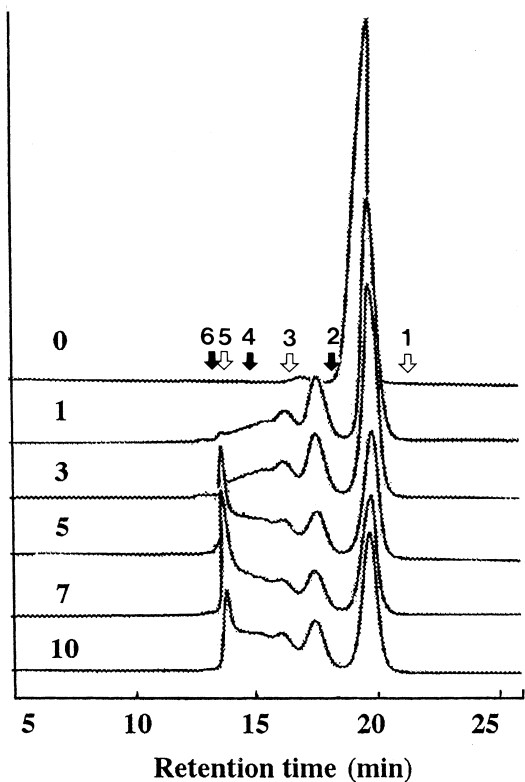


Fig. 2. Gel filtration patterns of dry-heated ovalbumin at 80°C for various periods of time. The numbers in the figure show heating time (days). Arrows indicate retention time (min) of standard proteins (1, myokinase, 32 kDa; 2, enolase, 67 kDa; 3, lactate dehydrogenase, 142 kDa; 4, glutamate dehydrogenase, 290 kDa; 5, thyroglobulin, 669 kDa; and void volume (6, blue dextran)).

Table 1
Surface hydrophobicity (S_0) of dry-heated OVA at 80°C for various periods of time

Heating time (days)	S_0	Heating temperature (°C)	S_0^a
0	30	75	330
1	40	80	1100
3	70	85	1400
5	100		
7	150		
10	145		

^a S_0 values of non-treated OVA solution (0.1%, pH 7.0) heated to the indicated temperature.

smaller molecular weight than OVA monomer were detected in the samples dry-heated over 3 days, although the intensities of their bands were faint (Fig. 3B, 3C). The result suggests the possibility of hydrolysis of peptide bonds in OVA molecules.

Table 1 shows surface hydrophobicity of OVA samples dry-heated at 80°C for various periods of time. The surface hydrophobicity gradually increased with increased heating time in the dry state. When dry-heated at 80°C for 7 day, the value of surface hydrophobicity was increased five-fold over that of non-heated OVA (0-days). This result indicates that the dry heating of freeze-dried OVA powder caused some structural changes with the exposure of the buried hydrophobic residues in OVA molecules. However, the values of hydrophobicity of the dry heat-treated OVA samples were much less than those of the untreated OVA heat-denatured in solution. This indicates that the dry heating treatment might cause more mild conformational changes in OVA when compared to the case of heating in solution.

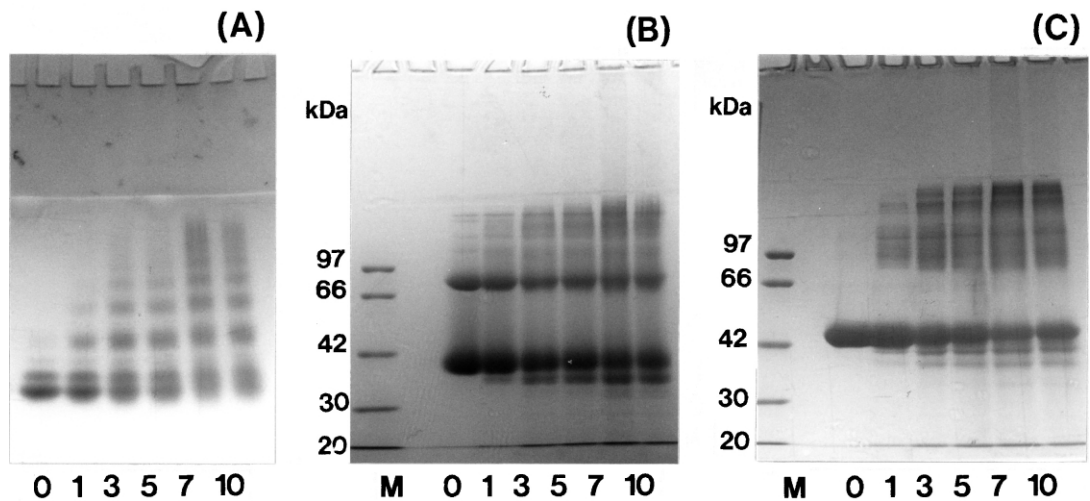


Fig. 3. Electrophoretic patterns of dry-heated ovalbumin at 80°C for various periods of time. (A) native-PAGE; (B) SDS-PAGE without 2-mercaptoethanol (ME); (C) SDS-PAGE with ME. The lane numbers show heating time (days). Lane M indicates marker proteins (phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; aldolase, 42 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa).

Native OVA contains four sulfhydryl (SH) groups and one disulfide (SS) group per molecule. The SH residues in OVA exist in the interior of the protein molecules and are exposed with heat denaturation (Matsudomi, Yamamura, & Kobayashi, 1986). Fig. 4 shows the changes of total and surface SH groups in OVA samples heated in the dry state for various periods of time. The total SH value of non-heated OVA (0-day) was 3.5 mole per mole of the protein, a little lower than the value (about 4 mole) of the native OVA reported (Nisbet et al., 1981). This suggests that some of the SH residues of OVA had been oxidized by the formation of

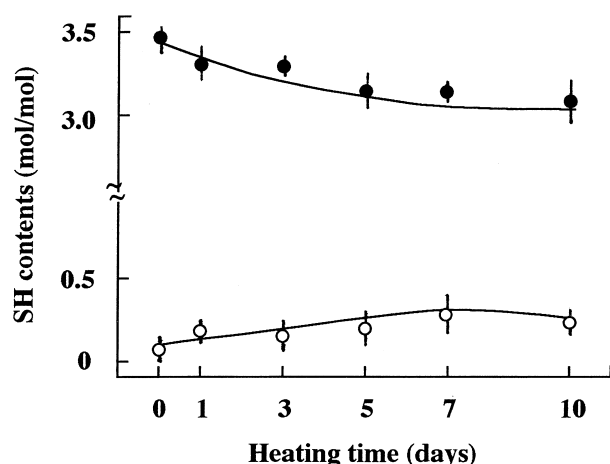


Fig. 4. Changes of total and surface sulfhydryl (SH) groups in dry-heated OVA at 80°C for various periods of time. (●) total SH; (○) surface SH. Data are the average of triplicate measurements.

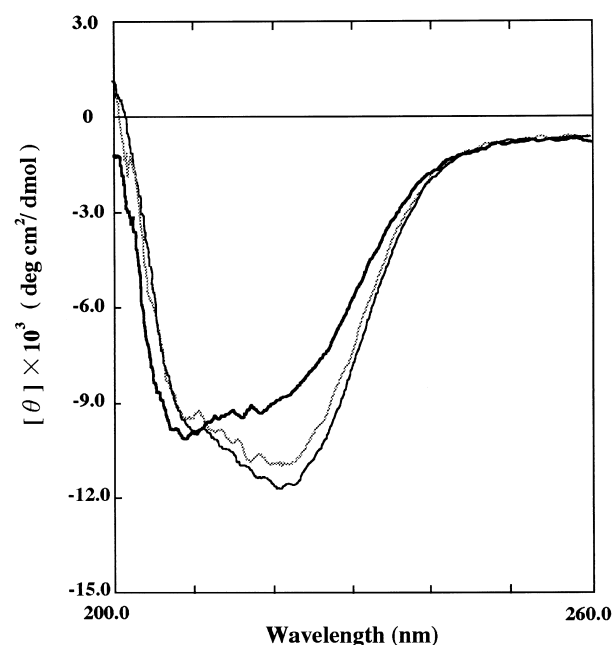


Fig. 5. Circular dichroism spectra of dry-heated, heat-denatured OVAs. (—) non-heated; (---) dry-heated for 7 days; (- - -) heat-denatured to 80°C at pH 7.0.

SS bridge in intra- or inter-molecules during storage at 4°C pending analysis. The total SH groups of OVA samples were gradually decreased with increased heating time. On the other hand, the surface SH groups in OVA samples were gradually increased with increased heating time. These results suggest that exposure of SH groups to the surface on OVA molecules and then SH-SS exchange reactions and SH oxidation might proceed simultaneously during heating in the dry state, which resulted in initial polymerization due to SH oxidation together with hydrophobic interaction.

The effects of the dry heating on the secondary structure of OVA were studied by CD spectra. As shown in Fig. 5, the CD spectra indicated that the level of organization in OVA and dry-heated OVA are nearly the same even after the dry heating at 80°C for 7 days, while that of OVA heat-denatured to 80°C in solution showed drastic changes in secondary structure. Native OVA is known to have 31% α -helix, 27.5% β -sheet, 13.5% β -turn, and 28% random coil (Batra, Roebuck & Utrecht, 1990). α -Helical content (α H) can be estimated from the mean residue ellipticity at 222 nm (θ_{222}), using the following equation

$$\alpha H = (\theta_{222} - \theta_c) / (\theta_h - \theta_c) \quad (1)$$

where θ_h and θ_c are the mean residue ellipticity values for fully helical and random coil polypeptides, respectively. Using the values of $-36,000$ and -260 in degree $\text{cm}^2/\text{d mol}$ for θ_h and θ_c , respectively (Chen, Yang, & Martinez, 1972; Greenfield & Fasman, 1969) α H for

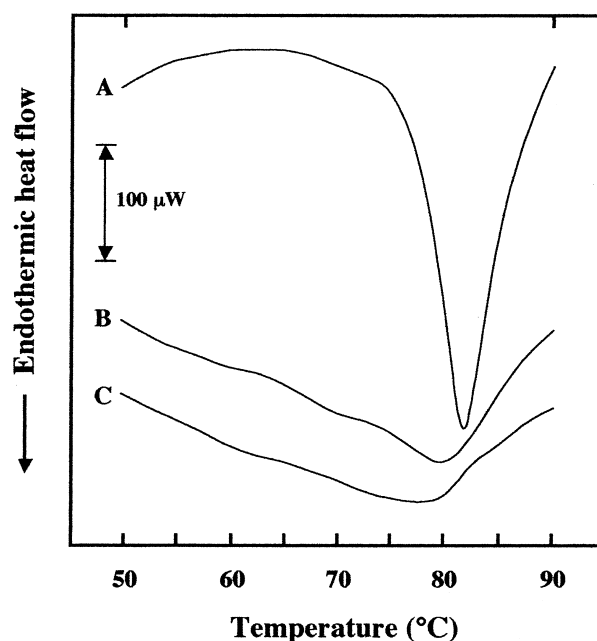


Fig. 6. Differential scanning calorimetry thermograms of dry-heated ovalbumin at 80°C for various periods of time. (A) non-heated; (B) dry-heated for 5 days; (C) dry-heated 7 days.

non-heated OVA (0-day) and dry-heated OVA (7 days) was obtained approximately as 31 and 29.5%, respectively. On the other hand, α H for heat-denatured OVA in solution at 80°C was about 23.5%. Thus, it is of interest that the dry heating at 80°C of OVA hardly affects the gross secondary structure, as measured by the helix content. Basically similar results were shown in the CD spectra of egg white proteins heated in a dry state (Kato et al., 1989; Mine, 1996).

DSC thermograms of OVA and dry-heated OVA samples are shown in Fig. 6. Non-heated OVA showed a sharp peak with a Td values of 80.1°C and an enthalpy of 13.8 J/g (similar values reported by Donovan, Mapes, Davis, & Garibaldi, 1975). The thermogram of dry-heated OVA sample for 7 days at 80°C showed more broadening of the endothermic peak with a detectable decrease in Td (78°C) and a marked decrease in enthalpy (10.6 J/g). It has been demonstrated that broadening of peak indicates the existence of denatured intermediates different from the native form (Myers, 1990; Wright, Leach, & Wilding, 1977). Therefore, this result suggests that the dry heating at 80°C changed native OVA to the unfolded state and led to a marked reduction of the thermal stability. However, considering the slight change in the CD spectrum and in surface hydrophobicity, the gross structural changes in OVA molecules occurring by the dry heating were thought to be very mild. Thus, partially unfolded conformations formed by the dry heating may play an important role during the subsequent heat for gelation, probably by promoting the formation of soluble aggregates through more extensive intermolecular interaction to induce firmer, more uniform OVA gels. It has been demonstrated by our previous paper that dry heating of OVA led to a decrease in the molecular weight of soluble OVA aggregates, and promoted the formation of aggregates

with expanded structure during the subsequent heat for gelation (Matsudomi et al., 1993).

The structural changes of OVA were followed by its adsorption behavior on an anionic exchange resin (DEAE-Toyopearl). As shown in Fig. 7, non-heated OVA was eluted with a very sharp peak, while dry-heated samples showed a broadening of peak in the elution pattern, indicating the higher affinity for the resin with increased heating time in the dry state. This behavior may reflect an increase of negative charge at surface of OVA molecules, and predicts that the dry heating would facilitate the exposure of a greater number of negative charges to the protein surface. Zhang, Lee and Ho (1993) documented that the thermal deamidation of some proteins occurred in a restricted water environment at 115°C for 2 h. Also, Mine (1997) recently studied the effect of pH during the dry heating at 75°C of egg white proteins, and reported that the dry heating of egg white proteins at mild alkaline pH (under 9.5) enhanced the degree of deamidation with increased heating time. The deamidation of proteins leads to an increase in the negative charge of proteins. The present study revealed that the dry heating at 80°C might cause an increase in negative charge on the protein surface (Fig. 7). Subsequently, the degrees of deamidation of dry-heated samples were determined from the amounts of ammonia generated by the reaction, as described in Section 2. As shown in Fig. 8, a steep increase of deamidation was observed in OVA sample dry-heated at 80°C for 1 day and the value of deamidation reached 6%, after that it tended to increase with increase of the dry heating and reached 12% after 10-days. Interestingly, in spite of the enhancement of denaturation and polymerization of OVA with increasing dry-heating time, a loss of water solubility of the protein was very small. This observation might be due to the effect of the

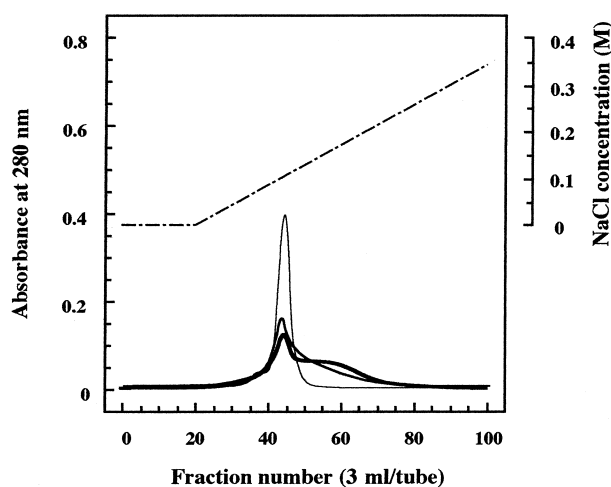


Fig. 7. Elution patterns of dry-heated ovalbumin at 80°C for various periods of time on a DEAE-Toyopearl column. (—) non-heated; (---) dry-heated for 5 days; (·····) dry-heated for 7 days.

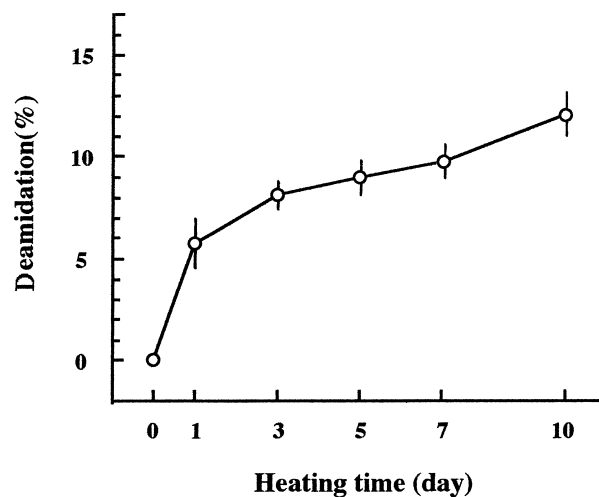


Fig. 8. Degree of deamidation of dry-heated ovalbumin at 80°C for various periods of time. Data are the average of triplicate measurement.

higher electrostatic repulsion as a result of deamidation of glutamine and asparagine residues in proteins (Matsudomi, Kato, & Kobayashi, 1985).

It has been reported that the gelling properties of protein are affected by the aggregation state of soluble aggregates formed during heat-induced gelation (Nakamura, Sugiyama, & Sato, 1978). Kitabatake, Hatta, and Doi (1987) reported a preparation of harder and transparent gels from OVA solution by a two-step heating method, and stated that the soluble aggregates formed from OVA when heated without salt produce such OVA gels even at high salt concentrations upon the subsequent heating (reheating) for gelation. In addition, Kato et al. (1990b) and Matsudomi et al. (1993) documented using egg white proteins and OVA, respectively that the dry heating of these proteins induced the formation of specific soluble aggregates of a low molecular weight and a homogeneous molecular size upon the subsequent heating for gelation, and that such soluble aggregates construct firm and transparent gel matrix. Thus, the properties of OVA gels prepared by a two-step heating method seemed to be considerably similar to those of gels from the dry-heated OVA. This suggests that mild heat treatments (heat denaturation without salt or dry-heating) of OVA prior to gelation would be required for the formation of specific soluble aggregates responsible for the construction of harder and transparent OVA gels.

In conclusion, it can be assumed that the partial denatured and deamidated OVA molecules derived from the dry heating, form the specific soluble aggregates under the balance of electrostatic repulsion and hydrophobic interaction during subsequent heat for gelation, which construct an ordered network to form transparent and firm OVA gels. In addition to protein deamidation, the dry heating was also found to cause the partial peptide cleavage of OVA molecules as observed in the SDS-PAGE analysis. The phenomenon may also affect the aggregation state of soluble aggregates formed during gelation. Study on the cleavage site of hydrolysis and the properties of monomeric fraction in dry-heated OVA molecules is in progress.

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