56-87-1; L-Asp, 56-84-8; L-Glu, 56-86-0; L-Ser, 56-45-1; L-Thr, 72-19-5; L-Tyr, 60-18-4; L-His, 71-00-1; L-Pro, 147-85-3; L-Arg, 74-79-3; Gly, 56-40-6; NaOH, 1310-73-2.

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Effect of Pre Heat Treatment on Tryptic Hydrolysis of Maillard-Reacted Ovalbumin

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Tryptic hydrolysis of a pre-heat-treated ovalbumin–glucose complex (OVG) was compared with that of pre-heat-treated ovalbumin (OV). The ovalbumin–glucose (7:3 in weight) mixture was stored at 50 °C and 65% relative humidity for a defined time (2–10 days), solubilized, and then freeze-dried. The level of hydrolysis after preheating of samples was measured with two methods, that is, determination of trichloroacetic acid precipitable protein concentration and calculation of ratio in unhydrolyzed and hydrolyzed fraction areas on a Sephadex G-75 column chromatographic pattern. Stored OVG hydrolysis was better than for OV and unstored OVG, although the number of free amino groups in stored OVG decreased with storage. Stored OVGs were irreversibly unfolded by heating (100 °C, 10 min) as indicated in the CD spectra but almost never aggregated to a high particle weight compound like the heat-treated OV. These results suggest that good tryptic hydrolysis of stored OVG depends on a heat-denatured conformation of OVG and reduced formation of aggregates.

The nutritional problems with food proteins induced by the Maillard reaction have been well investigated (Hurrell and Carpenter, 1977, 1981). The reaction is conventionally divided into early, advanced, and final stages (Hodge, 1953;

Ellis, 1959; Reynolds, 1963, 1965; Mauron, 1981) which induce a different type of nutritional damage. In the stage after the early Maillard reaction, it is well-known that the formation of colored and high cross-linking protein–carbohydrate polymers having high particle weight causes a lowering of solubility, digestibility, and nutritional value of the reacted proteins (Mauron, 1970; Clark and Tannenbaum, 1974). On the other hand, different results are obtained from the nutritional estimation of protein in the early Maillard reaction. The amino–carbonyl condensation

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product formed is rapidly converted via Schiff's bases to Amadori compounds. However, in heated milks 2.5–10% of the lysine has been reported to be present in Schiff's base form, which appeared to be biologically available to rats (Finot et al., 1977). The N^{ϵ}-(1-deoxy-D-fructosyl)-L-lysine residue, an Amadori product which is the main substituted lysine derivative form of the protein–glucose system, appeared to be released during digestion in vivo, absorbed, and then excreted in the urine under the form of unavailable peptide (Ford and Shorrock, 1971; Pronczuk et al., 1973). Thus, chemical properties of proteins reacted with reducing sugars and the like are important factors for digestibility and nutritional value.

In previous studies, the present authors found that the ovalbumin–glucose complex occurring in the early Maillard reaction possessed specific physicochemical properties such as high heat stability and solubility. Such a physical attributes of proteins might affect the digestibility.

It is well-known that native ovalbumin resists tryptic hydrolysis on the basis of its rigid conformation (Shmakova and Kaverzneva, 1969), whereas the heat-denatured substance is hydrolyzed (Romanoff and Romanoff, 1949). This study describes the effect of pre heat treatment on tryptic hydrolysis of Maillard-reacted ovalbumin, comparing with that of ovalbumin, and discusses the relationship between heat denaturation–aggregation and hydrolysis of such a complex.

EXPERIMENTAL SECTION

Material. Freeze-dried ovalbumin (OV) and ovalbumin containing D-glucose corresponding to 30% of the dry weight of the protein (OVG) were stored at 50 °C and 65% relative humidity for a defined time (2–10 days). The unstored and stored samples were solubilized in distilled water and dialyzed against the water and then freeze-dried again.

Pre Heat Treatment. Pre heat treatment of the prepared OV and OVGs solubilized in phosphate buffer (pH 8.0, $I = 0.1$) at 0.1% (w/v) was conducted by placing 3-mL samples in test tubes (1.3 × 10 cm), which were immersed in a controlled-temperature water bath. The solutions were kept at various temperatures (70 ± 0.5, 80 ± 0.5, 90 ± 1.0, and 99 ± 1.0 °C) for 10 min after reaching to the desired temperature. Each sample was cooled immediately after heat treatment by placing the tubes in ice water.

Tryptic Hydrolysis. The pre-heat-treated OV and OVG solutions were treated at 38 °C for 2 and 24 h with trypsin [porcine pancreatic trypsin (type XI) with 7150 BAEE units/mg of protein, Sigma Chemical Co.) at a 1:20 enzyme:substrate ratio.

Determination of Hydrolysis Level. Two methods were used to determine the level of OV and OVG hydrolysis by trypsin. In the first method, aliquots of 1 mL were removed from the digest at 2 and 24 h, and the reaction was quenched by the addition of 1 mL of 10% trichloroacetic acid (TCA) and then allowed to stand overnight at 5–6 °C. The samples were centrifuged at 900g for 15 min, and the obtained precipitates were dissolved in 2 mL of 0.1 N NaOH–10% Na_2CO_3. Protein concentrations in these solutions were determined by the Lowry method (1951). The extent of hydrolysis was calculated as

hydrolysis (%) = (total protein concentration –
TCA-precipitable protein)/total protein concentration
$$\times\ 100$$

In the second method, a Sephadex G-75 column (1.5 × 20.5 cm) was used for gel filtration experiment with phosphate buffer (pH 8.0, $I = 0.1$) at 5 °C to compare the peak areas

Table I. Hydrolysis Level (Percent)[a] of Pre-Heat-Treated OV and OVG by Trypsin after 24 h of Incubation[b]

	temperature of pre heat treatment, °C				
	unheated	70	80	90	100
OV-0	0	6	26	39	68
OV-8	0	6	25	35	69
OVG-0	0	7	25	33	68
OVG-3	0	23	36	62	69
OVG-8	0	37	57	61	70

[a] Each value is the mean from two samples. [b] OV-0, unstored OV; OV-8, 8-day-stored OV; OVG-0, unstored OVG; OVG-3, 3-day-stored OVG; OVG-8, 8-day-stored OVG.

measured at 280 nm with a UV spectrophotometer on the samples hydrolyzed for 2 and 24 h described above.

Polyacrylamide Gel Electrophoresis. A vertical flat-sheet polyacrylamide gel electrophoretic method (Reid and Bieleski, 1968) was used to separate the unstored and stored OV and OVG before and after pre heat treatment (7.5% polyacrylamide) and their hydrolysate with trypsin (7.5 and 15% polyacrylamides for 2- and 24-h incubations, respectively). Gel sheets (0.1 × 13 × 13 cm) and electrophoresis buffer of Tris–glycine were prepared as described by Davis (1964). Electrophoresis using a constant quantity of each sample was performed at room temperature with a constant of 20 mA for 3 h by using a discontinuous buffer system. The gel sheets were stained with a solution of 0.2% Coomassie Brilliant Blue R-250 in water-propanol–acetic acid (5:5:1 v/v/v) and destained by 7% acetic acid overnight.

Measurement of Free Amino Group Content and CD Spectrum. These were carried out essentially as described in previous reports (Kato et al., 1981a; Watanabe et al., 1980), and in measurement of free amino group content, α-pentylamine (Waco Pure Chemical Industries, Ltd., ultrapure grade) was used as a standard (Böhlen et al., 1973).

RESULTS

Various samples of unstored and stored OV and OVG were preheated at various temperatures in order to determine their effects on tryptic hydrolysis from TCA-precipitable protein concentrations. The obtained results are shown as hydrolysis levels in Table I. No unheated samples, even 8-day-stored OVG which was partially denatured as described previously (Watanabe et al., 1980), were hydrolyzed even after 24 h of digestion. On the other hand, the hydrolysis level in every pre-heat-treated OVG was increased by increasing the temperature. Higher percentages were obtained from lower temperature treated OVG with longer storage, although 8-day-stored OV showed almost the same degree of hydrolysis as the unstored OV. The same degree of hydrolysis was also found in all OV and OVG samples after preheating at 100 °C. Therefore, 100 °C for 10 min was selected as the preheating condition in the following experiments.

Hydrolysis levels from the TCA-precipitable protein concentrations of unstored and stored OV and OVG after preheating followed by 2- and 24-h digestion are shown in Figure 1. The degree of hydrolysis with the OV system did not change throughout the storage period, showing about 40% for 2-h digestion and about 68% for a 24-h one. On the other hand, with longer storage, OVG hydrolysis decreased for 2-h digestion and increased slightly for 24-h digestion.

Gel filtration profiles of hydrolysates of unstored and stored OV and OVG after pre heat treatment followed by 2- or 24-digestion are shown in Figures 2 and 3. In both
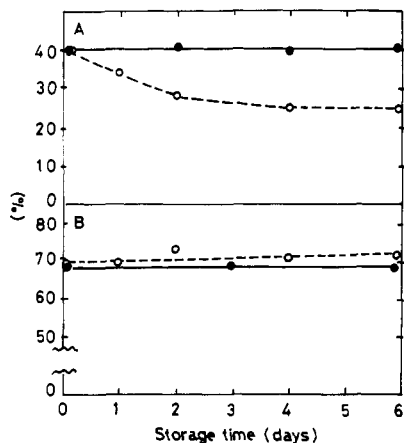
Figure 1. Level of tryptic hydrolysis of OV and OVG from the TCA-precipitable protein concentration. Preheat conditions: 100 °C; 10 min. (A) After 2-h digestion; (B) after 24-h digestion. (●) OV system; (○) OVG system. Each value is the mean from two samples.
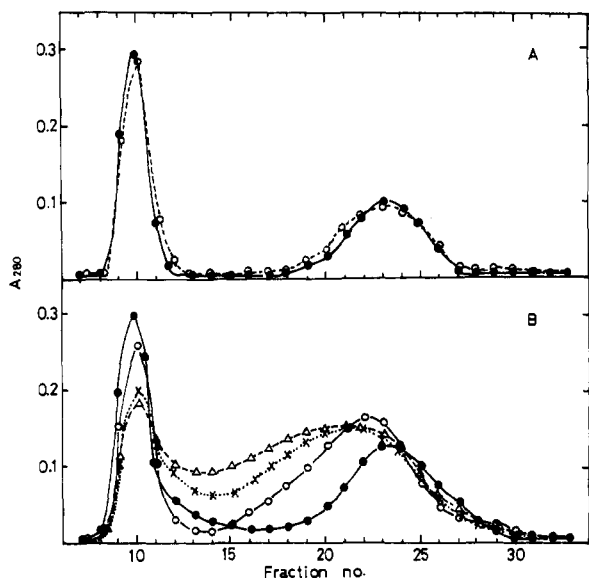

Figure 2. Gel filtration chromatographic analysis of tryptic hydrolysate of OV and OVG after 2-h incubation. Preheat conditions: 100 °C; 10 min. (A) OV system: (●) unstored OV; (○) 6-day-stored OV. (B) OVG system: (●) unstored OVG; (○) 2-day-stored OVG; (×) 4-day-stored OVG; (△) 6-day-stored OVG.

figures, the peak in fraction no. 7–13 and that in fraction no. 14–33 indicated unhydrolyzed protein and hydrolyzed peptides, respectively. The proportions of both areas are shown in Table II The unstored and stored OV showed almost the same proportion, whereas the effect of the storage length on OVG digestion was clearly indicated by the increase of hydrolyzed concentration as storage was longer.

The degree of hydrolysis in Figure 1 can be taken to be equivalent to the proportions of fraction no. 14–33 in the corresponding samples. However, except for unstored OV and OVG and 6-day-stored OV, the former showed less digestion than the latter. This difference could be explained from the fact that the TCA-soluble part was composed of fraction no. 22–33. Thus, from the estimation with gel filtration profiles the OVG system was clearly more easily hydrolyzed than the OV one, although the peptide fragments consisted of hydrolysates of the larger and smaller molecular weight.

In order to estimate the reason of high tryptic hydrolysis of pre-heat-treated OVG, the effect of pre heat treatment
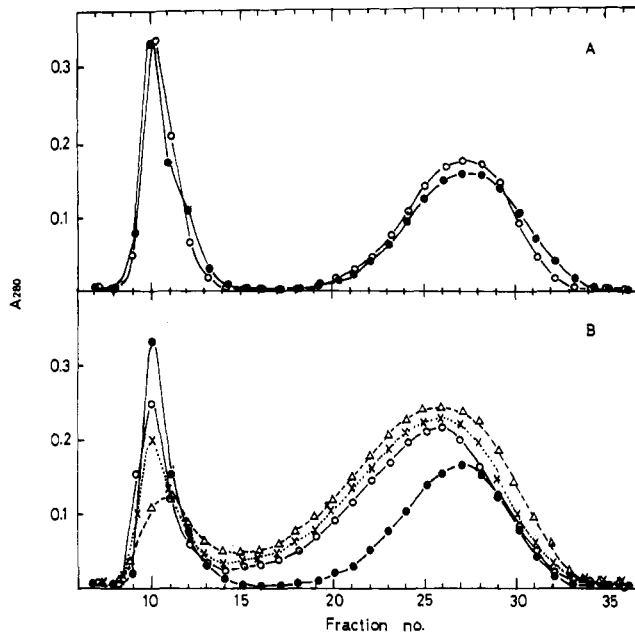

Figure 3. Gel filtration chromatographic analysis of tryptic hydrolysate of OV and OVG after 24-h incubation. Preheat conditions: 100 °C; 10 min. (A) OV system; (B) OVG system. Symbols are the same as indicated in Figure 2.
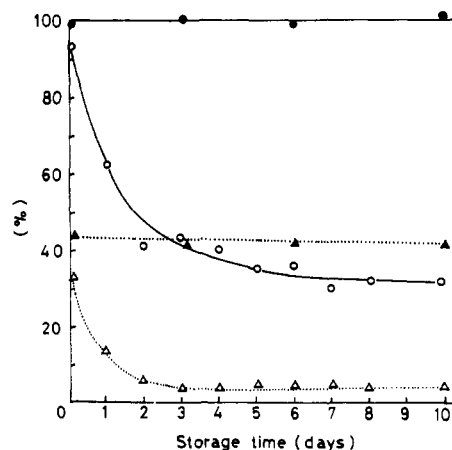

Figure 4. Remaining ratio of free amino group contents in OV and OVG before and after pre heat treatment (100 °C, 10 min). (●) OV after pre heat treatment; (○) OVG after preheat treatment; (▲) OV before pre heat treatment; (△) OVG before pre heat treatment. Each value is the mean from two samples.

Table II. Proportion of Unhydrolyzed and Hydrolyzed Parts by Trypsin after 2 and 24 h of Incubation[a]

	porportion of peak area, %[b]			
	2-h incubation fraction no.		24-h incubation fraction no.	
	7–13	14–33	7–13	14–33
OV-0	51	49	39	61
OV-6	50	50	38	62
OVG-0	41	59	36	64
OVG-2	30	70	25	75
OVG-4	29	71	21	79
OVG-6	26	74	15	85

[a] OV-0, unstored OV; OV-6, 6-day-stored OV; OVG-0, unstored OVG; OVG-2, 2-day-stored OVG; OVG-4, 4-day-stored OVG; OVG-6, 6-day-stored OVG. [b] Each value is the mean from two samples.

on the remaining free amino groups and denaturation of ovalbumin was examined with free amino group and CD analyses.
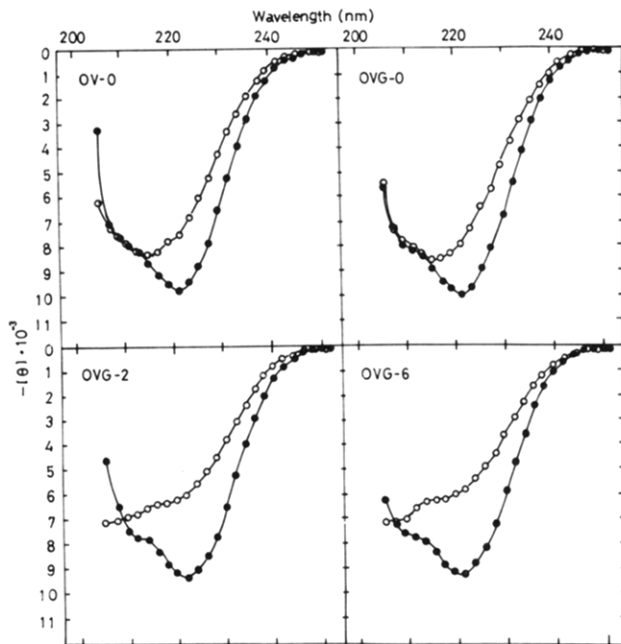
Figure 5. CD spectra of OV and OVG before (●) and after (○) pre heat treatment (100 °C, 10 min) in pH 8.0 phosphate buffer. OV-0, unstored OV; OVG-0, unstored OVG; OVG-2, 2-day-stored OVG; OVG-6, 6-day-stored OVG.

The free amino group contents measured by fluorometric assay using fluorescamine are shown in Figure 4. According to the method of Böhlen et al., all free amino groups in native protein should be reacted with fluorescamine. But free amino group contents in OV heated at 100 °C for 10 min more than doubled those of unheated OV. Consequently, free amino group contents in OV and OVG before and after pre heat treatment (100 °C, 10 min) are expressed as a percentage against that in heated OV. The free amino group contents in the OV system did not change with storage time, whereas those in the OVG system decreased with storage, as indicated in the OVG after heat treatment which decreased to about 50% after 2 days and reached about 30% after 10 days of storage.

CD spectra of unstored OV and OVG and OVG stored for 2 and 6 days before and after pre heat treatment at 100 °C for 10 min are illustrated in Figure 5. Every unheated samples showed almost the same CD pattern, but it was changed to its own pattern by preheating, showing greater denaturaion in 2- and 6 day-stored OVGs.

Polyacrylamide gel electrophoretic profiles of unstored and stored samples of OV and OVGs are shown in Figure 6. In the OV system and unstored OVG patterns, oval-bumins A_1, A_2, and A_3 with some contaminants and/or some aggregates of ovalbumin could be found. Due to the changes of charge groups on the protein surface by the attachment of glucose, the main components in stored OVG were migrated to more anode sites than ovalbumin A_1. Moreover, their samples contained some aggregates with much lower mobilities of ovalbumin A_3.

Polyacrylamide gel electrophoretic profiles of unstored and stored OV and OVG preheated at 70, 80, 90, and 100 °C for 10 min, respectively, are shown in Figure 6. With greater heating, the OV system and unstored OVG aggregated more than stored OVG, especially above 80 °C.

Polyacrylamide gel electrophoresis on the 2- and 24-h-hydrolyzed samples after pre heat treatment was carried out in order to determine in the presence of residual aggregates and peptide fragments. Their profiles with unhydrolyzed samples are illustrated in Figure 7. The profiles in the OV system and unstored OVG after 2-h digestion showed the presence of some hydrolysates with high particle weight aggregates which slightly entered into separation gel. And after 24-h hydrolysis of their samples, although the aggregates still remained, a large part of such peptide fragments could not be found, because they were probably so small that they moved to the front of the gel. In the stored OVG system, the widespread occurrence of peptide fragment instead of aggregates was observed.

DISCUSSION

Heated egg white is well-known to be more easily digested by man than an unheated one (Morgan et al., 1951). An it has been demonstrated in vitro that the tryptic digestibility of freeze-dried egg white heated at 100 °C for several minutes was better than with an unheated one. Similar results have been reported for other proteolytic enzymes such as pepsin, pancreatin, and Pronase (Scudamore et al., 1949). This good digestibility of heated egg white might depend on the denaturation of egg white proteins and inactivation of proteolytic inhibitor in egg white.

In the present results, all unheated samples were not hydrolyzed with trypsin, possibly because OV and OVG have the compact structure shown in CD spectra of unheated proteins (Figure 5). On the other hand, ovalbumin may have the tryptic inhibitory activity pointed out by Hunt and Dayhoff (1980), since the amino acid sequence of ovalbumin, antithrombin III, and α-proteinase inhibitor are significantly homologous. However, such trypsin inhibitory in OV has not been clearly substantiated. Consequently, the tryptic hydrolysis of OV and OVG would appear to depend on both the available lysine contents and the ovalbumin structure.
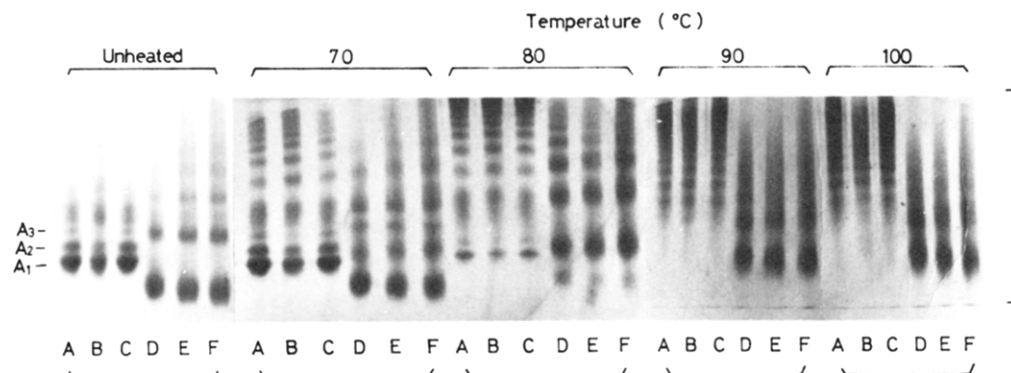

Figure 6. Effect of preheating temperature on the electrophoretic patterns of OV and OVG. Sample solutions (pH 8.0 phosphate buffer) were heated for 10 min at various temperatures. (A) Unstored OV; (B) 6-day-stored OV; (C) unstored OVG; (D) 2-day-stored OVG; (E) 4-day-stored OVG; (F) 6-day-stored OVG; A_1, A_2, and A_3, ovalbumin.

Tryptic Hydrolysis of Maillard-Reacted Ovalbumin

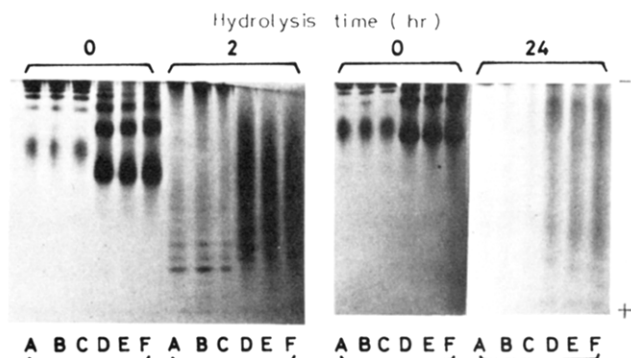*J. Agric. Food Chem.*, Vol. 31, No. 2, 1983 **441**

Figure 7. Electrophoretic patterns before and after tryptic hydrolysis of OV and OVG after pre heat treatment. Gel concentration: 7.5% for 2-h hydrolysate and its control; 15% for 24-h hydrolysate and its control.

The degree of OV and OVG hydrolysis showed that heated OVG, either unstored or stored, was hydrolyzed with trypsin at a higher level than corresponding OV samples, although the decrease of free amino groups in the OVG system inhibited the tryptic hydrolysis. And the stored OVG was more easily hydrolyzed than the unstored one (Figures 2, 3, and 7). One of the reasons for the high stored OVG hydrolysis might be that the Maillard reacted substances in early stage is reversible. Consequently, under the pre heat treatment, it was stipulated that the Maillard-reacted glucose might be released from the OVG complex. However, the increase in the ratio of the detectable free amino group after pre heat treatment numbered about as many or less than that of OV (Figure 4). Therefore, it is reasonable to say that the glucose is not released from the OVG complex by pre heat treatment.

The free amino groups on the samples were measured by fluorometric assay, the quick and sensitive method reported by Böhlen et al. (1973). Moreover, only 8 residues were measured on native OV, though one molecule of OV has 20 lysine residues. It seems likely that all free amino groups in native OV cannot react with fluorescamine because of the compact steric structure of the protein. After pre heat treatment, 30% of free amino groups was determined; some 70% of the available lysine reacted with glucose in this experiment. Hurrell and Carpenter (1978) also reported that the protein heated with glucose showed only a 30% decrease in available lysine, so all available lysine is not damaged in the early Maillard reaction.

Therefore, the main reason for the good hydrolysis is as follows. The CD spectra of stored and pre-heat-treated OVGs were much different from the unheated OVG spectra, which showed almost the same pattern as the CD spectrum of OV. The stored OVG structures easily unfolded under pre heat treatment and/or did not refold easily after cooling.

The present authors have already reported that OVG was kinetically stable on heat denaturation (Kato et al., 1981b). However, the structure of heat irreversible denatured OVG would probably be more random than that of heated OV as revealed by the CD spectra of heated OVGs (Figure 5). Thus, the unstored conformation of heated OVG may be associated with its good hydrolysis.

The stored OVG did not form high weight aggregates even at a heating temperature of 100 °C, whereas such aggregates could be easily formed in OV and unstored OVG. A protective glucose layer and its bound water on the protein surface of OVG probably hindered the formation of high particle aggregates. Such a large particle seems disadvantageous in proteolytic hydrolysis because of its lower surface area per unit weight.

In conclusion, the present results suggest that good hydrolysis is based on the heat irreversible denatured conformation of OVG and reduced formation of high weight particle aggregates.

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