

## Characterization of Heat Denatured $\beta$ -Lactoglobulin: Impact of Cooling Rate on Refolding and Aggregate Formation

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### Abstract

A solution of  $\beta$ -Lactoglobulin ( $\beta$ -Lg) was heated and successively cooled for 2 h at room temperature (slow cooling) or in an ice bath (rapid cooling). The surface SH content and surface hydrophobicity of the heated protein, which increased upon heating, decreased to a lesser extent upon rapid cooling compared to slow cooling, showing that structural changes in thermally denatured  $\beta$ -Lg are suppressed by rapid cooling. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography showed no significant differences in aggregate formation from denatured  $\beta$ -Lg between the rapid and slow cooling conditions. On the other hand, near-UV circular dichroism (CD) spectra of heat-denatured  $\beta$ -Lg showed significant differences between the rapid and slow cooling conditions. These results imply that the rapid cooling suppressed protein refolding rather than aggregate formation, resulting in highly conserved surface SH content and surface hydrophobicity.

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**Key words** :  $\beta$ -lactoglobulin, heat denaturation, cooling rate, circular dichroism spectroscopy, refolding

### Introduction

Heating is an important part of food processing and cooking. Generally, food materials are heated before being eaten to increase digestibility, decrease toxicity such as that arising from bioactive proteins, and add specific flavors for increased palatability.

Heat treatment gelatinizes starch/water mixtures, melts fats and denatures proteins<sup>1)</sup>. In particular, proteins included in food materials such as egg white, milk and soybean have been reported to change into other forms, for example, soluble aggregates<sup>2-5)</sup>, precipitates<sup>6-8)</sup> and coagulate-like gels<sup>9-13)</sup> as a result of heat treatment.

Furthermore, it has been reported that cooling of heat-denatured protein solutions also affects the denaturation behavior of protein molecules. Cooling promotes the refolding of unfolded protein

molecules<sup>14,15)</sup>. Shimoyamada et al.<sup>16)</sup> reported that a gel-like coagulate, referred to as freeze-gel, was formed by successive heating, precooling and frozen storage of soymilk. In this process, precooling at  $-5^{\circ}\text{C}$  was essential for freeze-gelation of the soymilk. The precooling was reported to have two significant effects, namely, supercooling<sup>17)</sup> and rapid cooling<sup>18)</sup>. Rapid cooling maintained the higher surface SH content and surface hydrophobicity, which were increased by heating of the soymilk. These results were attributed to the higher reactivity of heat-denatured protein molecules<sup>18)</sup>, implying that the denaturation behavior of the protein was affected by cooling as well as heating. In order to elucidate the effects of cooling rate on the heat denaturation of other proteins, a model protein was subjected to heating and two kinds of cooling in this study.

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Bovine  $\beta$ -lactoglobulin ( $\beta$ -Lg), which is one of the major constituents in bovine milk whey, is a compact globular protein with a molecular weight of 36,700 as a dimer<sup>19</sup>.  $\beta$ -Lg and whey protein form a gel as a result of heating<sup>9,13</sup> or successive heating in a low-ionic-strength solvent, cooling and salt addition, for example, cold set gelation<sup>20,21</sup>.

The aim of this study was to estimate the effects of cooling rate on the denaturation behavior of heated  $\beta$ -Lg and compare the denaturation behavior with that of the soymilk protein. For this purpose, two temperatures were selected for cooling, namely, cooling at room temperature, which resulted in slow cooling, and cooling in an ice bath, which resulted in rapid cooling of the heated  $\beta$ -Lg solution. To estimate the denaturation behavior, surface SH content and surface hydrophobicity of heated and cooled protein were measured. Aggregates that formed upon heating were analyzed by SDS-PAGE and gel chromatography. In order to estimate the effect of cooling rate on the secondary and tertiary structures of the protein, CD spectra were also measured both in the far-UV and near-UV regions after cooling of the heated  $\beta$ -Lg.

## Materials and Methods

### Heat treatment

Bovine  $\beta$ -Lg (Sigma-Aldrich Co., St. Louis, MO) was dissolved in 0.1 mol/L phosphate buffer (pH 7.6). The resulting solution (concentration: 2 mg/mL) was divided into screw-capped test tubes (1 mL each) and heated in boiling water. Some of the heated samples were cooled in an ice bath (rapid cooling) and the others were cooled at room temperature (slow cooling).

### Protein surface SH content and surface hydrophobicity

The protein surface SH content was estimated by using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)<sup>22</sup>. The adequately diluted sample (2 mL) was mixed with 0.3 mL of  $1.0 \times 10^{-2}$  mol/L DTNB solution and incubated at room temperature for 20 min. The absorbance of the incubated solution was measured at 412 nm.

The surface hydrophobicity of  $\beta$ -lactoglobulin was measured using 8-anilino-1-naphthalene sulfonic acid (ANS)<sup>23</sup>. The sample (80  $\mu$ L) was diluted with 1920  $\mu$ L of 0.01 mol/L phosphate buffer (pH 7.6) and mixed with 10  $\mu$ L of  $8 \times 10^{-3}$  mol/L ANS solution. The mixture was analyzed with a fluorescence spectrophotometer (F-2500, Hitachi High-Technologies Co., Tokyo, Japan) to measure its fluorescence (excitation: 390 nm; emission: 470 nm). All measurements were performed in triplicate.

### Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12.5% gels according to the method reported by Laemmli<sup>24</sup>. The gels were stained with Coomassie Brilliant Blue R-250.

### High performance liquid chromatography (HPLC)

The aggregate derived from  $\beta$ -Lg upon heating was analyzed by HPLC with a gel filtration column (Superose 6 10/300 GL, GE Healthcare UK Ltd., Little Chalfont, England). A pump (510, Waters Co., Milford, MA), a dual-wavelength absorbance detector (2487, Waters Co.) and a Rheodyne 7125 injector were used for the HPLC analyses. The mobile phase was 0.01 mol/L phosphate buffer (pH 7.6) and the flow rate was 0.5 mL/min. The eluent was monitored by UV absorbance at 254 nm.

### Circular dichroism (CD) spectra.

The CD spectra of heated and cooled  $\beta$ -Lg were measured using a spectropolarimeter (J-820, JASCO Co., Tokyo, Japan). The  $\beta$ -Lg solution (0.1 mg/mL) was heated at 100°C for 5 min and cooled for 1 h at room temperature or in an ice bath. Cells having a path length of 0.1 cm and 1 cm were used in the far-UV and the near-UV measurements of the resulting protein solutions, respectively. All measurements were done at room temperature.

## Results and Discussion

### Effect of cooling rate on surface SH content and surface hydrophobicity of heated $\beta$ -Lg

In our previous paper<sup>18</sup>, we reported that rapid

cooling of heated soymilk is essential to form a freeze-gel from soymilk. The rapid cooling maintained higher surface SH content and surface hydrophobicity of proteins in the heated soymilk, and thus the reactivity between the proteins through SH -SS exchange and hydrophobic interactions was sufficiently high to form a three-dimensional structural network during frozen storage of the soymilk.

In this study,  $\beta$ -Lg was used in the heating and cooling experiments instead of soymilk proteins since  $\beta$ -Lg is a compact globular protein and thus more easily characterized than the soymilk proteins. Figure 1 shows the surface SH contents of  $\beta$ -Lg solutions that were heated in boiling water and subsequently rapidly cooled in an ice bath or slowly cooled at room temperature. The SH content of  $\beta$ -Lg increased upon heating and plateaued after 1 min. The SH content of  $\beta$ -Lg cooled in an ice bath was significantly higher than that of the sample cooled at room temperature. The increase in surface SH content as a result of heating is generally attributed to the formation of SH groups from the breaking of S-S bonds and the exposure of internally buried SH groups, which has been assigned to Cys121 in the case of  $\beta$ -Lg<sup>25)</sup>. During cooling, the surface SH content is thought to decrease due to the reformation the S-S

bonds or the return of the SH groups to the interior of the protein molecule. Rapid cooling appeared to suppress the reformation of intramolecular or intermolecular S-S bonds and/or the partial refolding of thermally unfolded  $\beta$ -Lg molecules. This result agrees well with our previous results for soymilk, where the surface SH content of soymilk protein was significantly higher in rapidly cooled soymilk than slowly cooled soymilk<sup>18)</sup>.

The protein surface hydrophobicity of the  $\beta$ -Lg solution, which was heated in boiling water and successively cooled in an ice bath or at room temperature, was also measured (Figure 2). The surface hydrophobicity was increased by heating for a short period of time, and longer periods of heating did not result in a large increase in surface hydrophobicity. Estimating the effect of cooling rate, the surface hydrophobicity of rapidly cooled  $\beta$ -Lg was slightly higher than that of slowly cooled  $\beta$ -Lg. These results were nearly identical to those observed for the soymilk protein<sup>18)</sup>.

As a result of heating,  $\beta$ -Lg was denatured and unfolded, which increased the surface hydrophobicity. It has been reported that the unfolded protein molecule refolds during cooling and the increased surface hydrophobicity decreases<sup>14)</sup>. The data shown

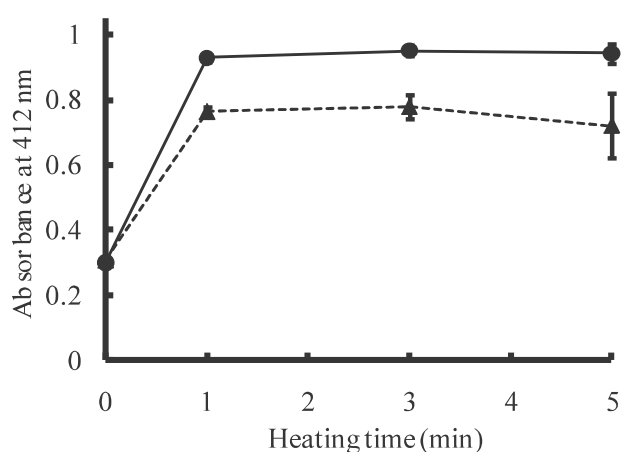


Fig. 1. Effect of cooling rate on surface SH content of heated and cooled  $\beta$ -Lg.

Solid line: rapidly cooled in an ice bath after heating; dotted line: slowly cooled at room temperature.

Mean  $\pm$  SD in triplicate experiments.

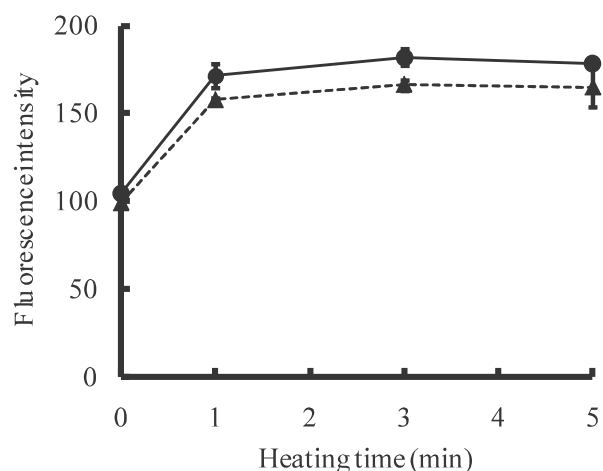


Fig. 2. Effect of cooling rate on surface hydrophobicity of heated and cooled  $\beta$ -Lg.

Solid line: rapidly cooled in an ice bath after heating; dotted line: slowly cooled at room temperature.

Mean  $\pm$  SD in triplicate experiments.

in Figure 2 suggest that rapid cooling suppressed refolding of thermally denatured  $\beta$ -Lg to maintain its surface hydrophobicity.

In the case of soymilk, changes in protein surface hydrophobicity were attributed to aggregate formation as well as unfolding and refolding of the protein<sup>5, 26</sup>.

### Aggregate formation of heated $\beta$ -Lg

The heated and cooled  $\beta$ -Lg solution was analyzed by SDS-PAGE to estimate the effect of cooling rate on aggregate formation, where protein molecules combine through disulfide bonds. SDS-PAGE without 2-mercaptoethanol showed that native  $\beta$ -Lg primarily consisted of monomer (Figure 3). After heating, the amount of monomer decreased and the amount of dimer increased along with the appearance of trimer- and tetramer-like constituents. These oligomers were expected to be formed through S-S bonds, because the samples with 2-mercaptoethanol showed only a monomer band. However, there was no clear

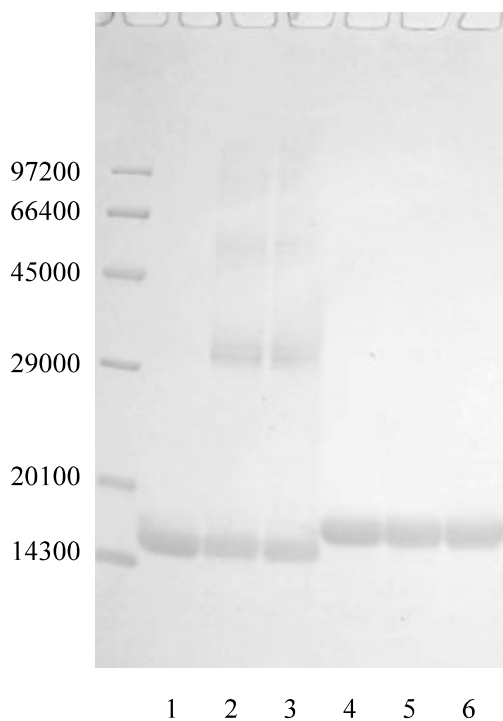


Fig. 3. SDS-PAGE patterns of heated and cooled  $\beta$ -Lg. Lanes 1, 4: non-heated; lanes 2, 5: slowly cooled; lanes 3, 6: rapidly cooled. Lanes 1, 2, 3: without 2-ME; lanes 4, 5, 6: with 2-ME.

difference in the composition of the oligomeric protein molecules between the rapidly and slowly cooled samples.

In order to detect aggregates formed through non-covalent bonds like hydrophobic interaction, the heated and cooled  $\beta$ -Lg was then analyzed by HPLC using a gel filtration column. The native  $\beta$ -Lg was observed mainly as a dimer (Figure 4), which was formed by a hydrophobic interaction. Heated  $\beta$ -Lg had a decreased dimer peak and increased aggregate peaks. In previous papers<sup>2, 4</sup>, it has been reported that large aggregates eluted at the void volume of the gel filtration column. In this study, only relatively small aggregates formed due to the low protein concentration. However, there were no significant differences between the rapid and slow cooling conditions. These results show that cooling rate does

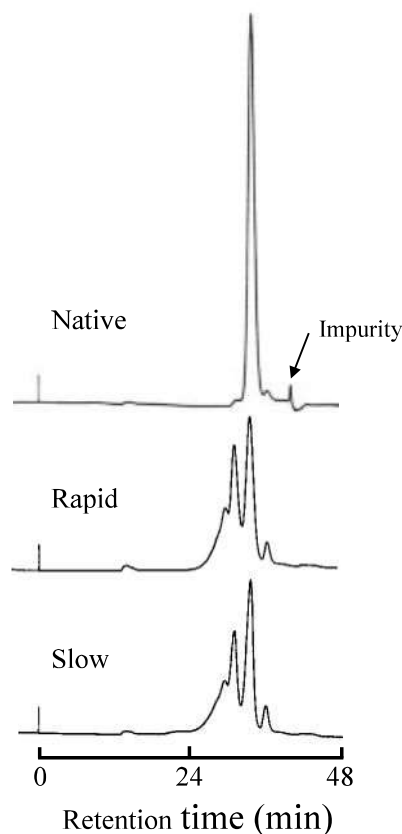


Fig. 4. Gel filtration chromatograms of heated and cooled  $\beta$ -Lg.

Native: non-heated  $\beta$ -Lg; Rapid: rapidly cooled in an ice bath after heating; Slow: slowly cooled at room temperature.

not markedly affect aggregate formation, including both covalently and/or non-covalently bound aggregates.

### Secondary and tertiary structures of heated and cooled $\beta$ -Lg

Rapid cooling suppressed the decrease in the surface SH content and surface hydrophobicity of the heat-denatured  $\beta$ -Lg, although the suppression was not related to aggregate formation. Therefore, in order to estimate the effect of cooling rate on the secondary and tertiary structures of thermally denatured  $\beta$ -Lg, CD spectra of the heated  $\beta$ -Lg solutions were measured after cooling at the two cooling rates (Figure 5). The CD spectra in the far-UV region, which provides information on secondary structure, for example,  $\alpha$ -helices and  $\beta$ -sheets<sup>27)</sup>, were clearly changed as a result of heating. A minimum at 216 nm shifted to 209 nm, indicating a random coil structure<sup>15)</sup>. Moreover, the ellipticity decreased, implying that changes in the secondary structure of  $\beta$ -Lg due to heat treatment coincided with those in a previous paper<sup>28)</sup>. However, there was little difference between the rapid and slow cooling conditions, and the secondary structure of  $\beta$ -Lg was almost equivalent regardless of the cooling conditions used.

On the other hand, the CD spectra in the near-UV region, which indicate the tertiary structure of a

protein by providing information on the local environment of aromatic amino acid residues, such as Trp, Tyr and Phe, were different for the two cooling conditions. The native  $\beta$ -Lg showed two deep troughs at 284 and 292 nm, which have been attributed to a Trp residue<sup>15, 28)</sup>. After heating at 100°C for 5 min,  $\beta$ -Lg cooled in an ice bath had a broad peak around 255 to 330 nm, and there were almost no troughs at 284 and 292 nm assigned. These data are almost equivalent to the spectra reported by Manderson et al.<sup>28)</sup>, in which  $\beta$ -Lg solution ( $\sim 3.5$  mg/mL) was heated at 86°C for 12.5 min and then rapidly cooled in ice flakes for at least 10 min. However,  $\beta$ -Lg cooled at room temperature showed much smaller troughs at 284 and 292 nm, and the ellipticity at 284 nm was lower than that at 292 nm, clearly differing from both of the native and the rapidly cooled  $\beta$ -Lg samples. The CD spectra obtained in this study showed that heat treatment changed the tertiary structure around Trp. The resulting unfolded protein molecules partially refolded during slow cooling; however, rapid cooling suppressed the refolding of  $\beta$ -Lg to maintain the unfolded form.

Manderson et al. reported that  $\beta$ -Lg which was heated at 86°C and then cooled in ice flakes, indicated loss of characteristic troughs in the CD spectra, and our experiment showed that  $\beta$ -Lg which was heated at 100°C and then cooled in an ice bath, showed

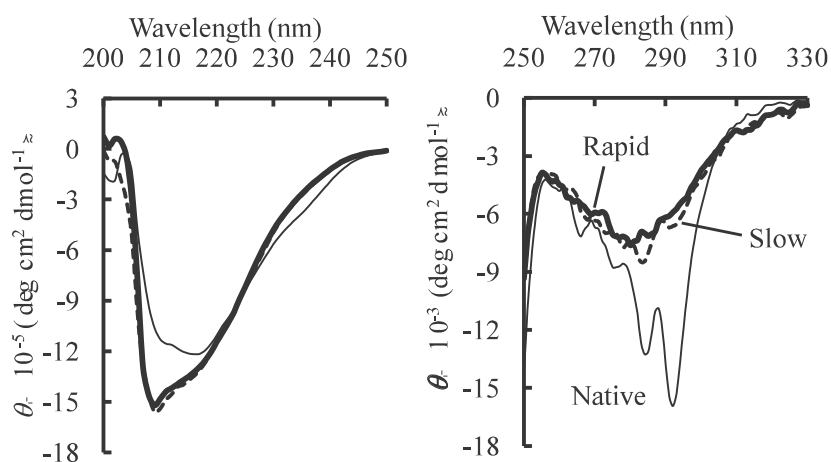


Fig. 5. CD spectra of heated and cooled  $\beta$ -Lg. Left: far-UV region; right: near-UV region. Solid line: Native, non-heated; bold line: rapidly cooled in an ice bath after heating; dotted line: slowly cooled at room temperature.

similar results. However,  $\beta$ -Lg cooled at room temperature showed some notable troughs, indicating some refolding of  $\beta$ -Lg during slow-cooling. These data may show that cooling condition (rapid or slow cooling) have equivalent or more significance on tertiary structure of  $\beta$ -Lg after heating comparing with heating condition.

The rapid cooling suppressed the decrease in the surface SH content and the surface hydrophobicity of heated  $\beta$ -Lg, and the result was almost equivalent to our previous study on soymilk protein<sup>18)</sup>. Considering the CD spectra, the suppression by rapid cooling may be due to suppression of partial refolding of heated  $\beta$ -Lg during cooling. On the other hand, there was no difference in aggregate formation of  $\beta$ -Lg through disulfide bonds and/or hydrophobic interaction. Based on all the above data, we conclude that the heat-denatured  $\beta$ -Lg protein partially refolded during slow cooling and that rapid cooling suppressed this partial refolding but not aggregate formation, when a dilute protein solution was used.

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## $\beta$ -ラクトグロブリンの加熱変性挙動の解析：リホールディングと会合体形成に及ぼす冷却速度の影響

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牛乳ホエータンパク質である $\beta$ -ラクトグロブリン溶液を加熱後、連続的に室温あるいは水中にて冷却した。タンパク質変性度の指標として用いられる表面SH基量と表面疎水性度は水中で急速に冷却した方が室温で緩慢に冷却した場合よりも高い値を示し、加熱直後の状態をより保っていた。SDS-PAGEとゲルろ過HPLCで会合体の形成について分析したところ、加熱によって生じる会合体の生成量には冷却速度の影響は見られなかった。そこで、CDを用いてタンパク質の高次構造変化について検討した。その結果、加熱によって近紫外領域の特徴的なシグナルはほぼ消失し、三次構造の一部は失われたものと考えられた。加熱溶液を緩慢に冷却すると再びシグナルが検出され、リホールディングが起こっているものと考えられた。一方、急速に冷却したタンパク質ではシグナルが観察されず、加熱変性によって三次構造の失われた状態が保たれているものと考えられた。以上の結果より $\beta$ -ラクトグロブリンを加熱後、冷却する際の速度は会合体形成よりはむしろリホールディングに対して大きく影響しているものと推測された。

**キーワード：**  $\beta$ -ラクトグロブリン, 加熱変性, 冷却速度, 円二色性スペクトル, リホールディング

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