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*Note*

# Enzyme Activity of Lysozyme-Dextran Complex Prepared by High Pressure Treatment

Kenjin NAKAMURA, Noboru FURUKAWA, Akiyoshi MATSUOKA, Tsuyoshi TAKAHASHI and Yoshitada YAMANAKA

*Department of Zootechnical Science, Tokyo University of Agriculture, Sakuragaoka, 1-chome, Setagaya-ku, Tokyo 156, Japan*

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**The relative activity of lysozyme by high pressure treatment over the pressure range of 50-300 MPa for 10 min at 0, 35 and 60°C was investigated. The activity gradually decreased by treatment at more than 200 MPa at 60°C, though stable at 0 or 35°C. To obtain a lysozyme with high activity, the lysozyme-dextran complex was prepared from a premixed solution of lysozyme and dextran, which was exposed to high pressure. The lysozyme-dextran complex showing the highest activity was prepared by the high pressure condition of 150 MPa for 60 min at 60°C in the weight ratio of 1 : 5 (lysozyme : dextran). It was concluded that the enhancement of the lysozyme activity of the lysozyme-dextran complex was due to the combination of lysozyme and dextran that was a hydrophilic polysaccharide.**

Keywords: bacteriolytic activity, high pressure treatment, lysozyme, protein engineering

Pressure treatment is an important operation for the chemical synthesis as well as temperature, concentration and pH. We aimed at the high pressure treatment being an available procedure for molecular structural modification such as protein engineering. Previous studies concerning the preparation of the lysozyme-saccharide complex by the Maillard reaction had not paid any attention to the formation of melanoidin, which reduced the solubility of the lysozyme-saccharide complex (Nakamura *et al.*, 1991; Kunugi & Tanaka, 1994). However, protein-saccharide complexes prepared by the Maillard reaction at high pressure did not have decreased solubility because of suppressing the formation of melanoidin (Tamaoka *et al.*, 1991). There was no report of the structural modification of lysozyme by high pressure treatment, though the effects of high pressure treatment on the bactericidal activities of egg white lysozymes have already been reported (Taniguchi *et al.*, 1994). The high pressure treatment was available for protein engineering, as it was reversible.

In this paper, the effects of high pressure treatment on the bacteriolytic activity of lysozymes were investigated. The lysozyme-dextran complex was then prepared by the high pressure treatment and its lytic activity determined.

## Materials and Methods

*Materials* Lysozyme (EC 3.2.1.17; M.W. 14,500 (Funatsu & Tsuru, 1977)) purified from hen egg white and recrystallized three times was purchased from Sigma Chemical Company (Missouri, USA). Dextran (M.W. 9,500) as a conjugating saccharide with lysozyme was from Pharmacia LKB (Uppsala, Sweden). *Micrococcus luteus* (ML) cell as a substrate for the determination of lysozyme activity was from Seikagaku Kougyou Co., Ltd. (Tokyo, Japan).

*Lysozyme activity* Lysozyme activity was estimated by the turbidity decrease in the ML cell suspensions (Smoelis & Hartsell, 1949). The turbidities were determined by absorbance at 540 nm with Hitachi 220A Spectrophotometer (Hitachi Co., Ltd. Tokyo, Japan).

*Lysozyme tolerance to high pressure treatment* The tolerance of lysozyme to high pressure treatment was estimated by the bacterial activity of 0.1% (w/v) egg white lysozyme-0.1 M phosphate buffer (pH 6.2) solutions, which were treated under high pressure conditions (50-300 MPa) for 10 min at 0, 35 and 60°C, using a high pressure generator for food processing (IHI-ITP type 70; Ishikawajimaharima Heavy Industry Co., Ltd., Tokyo, Japan) and cooled immediately in ice water. The measurement of lysozyme activities were carried out within 2 h.

*Preparation of lysozyme-dextran complex* To prepare the lysozyme-dextran complex, a mixture of powdered lysozyme and dextran in the weight ratio of 1 : 5 was dissolved to 0.1% (w/v) for a final concentration of lysozyme in 0.1 M phosphate buffer (pH 6.2). The premixed solution of lysozyme and dextran was treated under the high pressure of 150 MPa for 60 min at 60°C. The forming lysozyme-dextran complex was separated from the high pressure treated solutions by gel permeation chromatography (Fisher, 1969) using a Sephacryl S-300 column ($\phi$2.5×75 cm) (Nakamura *et al.*, 1991). The column was equilibrated and eluted with 0.1 M acetate buffer (pH 5.0) containing 50 mM sodium chloride. The protein in each fraction (5 g) was detected by measuring the absorbance at 280 nm, and the saccharide was determined by its color density at 490 nm developed with phenol-sulfuric acid (Dubois *et al.*, 1956). All fractions containing the lysozyme-dextran complex were collected together, dialyzed against filtered distilled water (FDW), concentrated by polyethylene glycol (M.W. 20,000) and diluted to 50 ml with FDW. This lysozyme-dextran complex was stored in a freezer

(−30°C) for the measurement of binding ratio and lysozyme activity.

*Relative lysozyme activity in lysozyme-dextran complex* The relative lysozyme activity in the lysozyme-dextran complex was expressed as follows;

$$\text{Relative lysozyme activity (\%)} = (LA_{\text{complex}})/(LA_{\text{lysozyme}}) \times 100$$

The $LA_{\text{complex}}$ denotes lysozyme activity in the lysozyme-dextran complex and the $LA_{\text{lysozyme}}$ denotes lysozyme activity in the egg white lysozyme.

*Binding ratio of lysozyme-dextran complex* The molar ratio of lysozyme to dextran in the lysozyme-dextran complex was estimated by the amounts of protein and saccharide in the lysozyme-dextran complex determined by the Lowry method (Lowry *et al.*, 1951) and phenol-sulfuric acid method, respectively.

## Results

*Lysozyme tolerance to high pressure treatment* Lysozyme activities were decreased by the high pressure treatment of more than 200 MPa at 60°C, though the activities were not decreased by the high pressure treatment at 0 and 35°C even at 300 MPa (Fig. 1). It was suggested that lysozyme activity was affected by the temperature during the high pressure treatment. From these results, the optimum pressure condition for the preparation of the lysozyme-dextran complex was considered to be about 150 MPa, which did not decrease their lysozyme activity.

*Preparation of lysozyme-dextran complex* Figure 2 shows the elution patterns of the separated fractions of the lysozyme-dextran mixture in the weight ratio of 1 : 5 (lysozyme : dextran) at 150 MPa for 60 min at 60°C by gel permeation chromatography using a Sephacryl S-300 column ($\phi$2.5×75 cm). In the non-treated lysozyme-dextran mixture, protein and saccharide were separate peaks. On the other hand, the peaks of protein by high pressure treatment were divided in two peaks, the first was small and the second was large. However there was only one peak for the saccharide. The small peak of protein was overlapped with that of the saccharide. The peak containing both protein and saccharide was confirmed as the fraction of the lysozyme-dextran complex, which was produced in the complex by high pressure treatment.

*Relative lysozyme activity in lysozyme-dextran complex* Figure 3 shows the relative lysozyme activity in the lysozyme-dextran complex determined by a decrease in the turbidity in

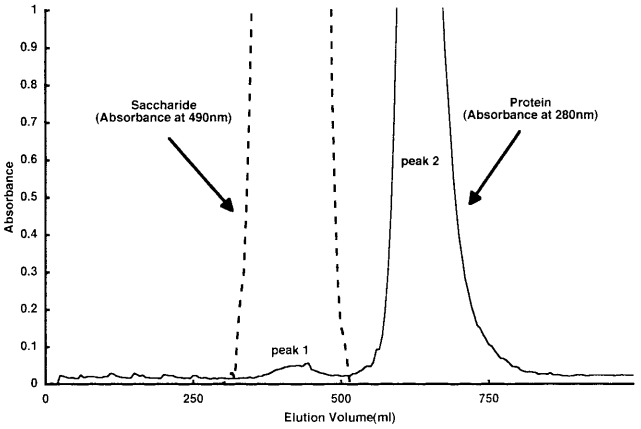


**Fig. 2.** Gel permeation chromatographic pattern of high pressure treated lysozyme-dextran mixture. The lysozyme-dextran solutions were treated at a pressure of 150 MPa for 60 min at 60°C. The lysozyme-dextran complex were separated by gel permeation chromatography using a Sephacryl S-300 column ($\phi$2.5×75 cm), and eluted with 0.1 M acetate buffer (pH 5.0) containing 50 mM sodium chloride. Absorbance at 280 nm for protein; absorbance at 490 nm to follow the color development using the phenol-sulfuric acid method for saccharide.

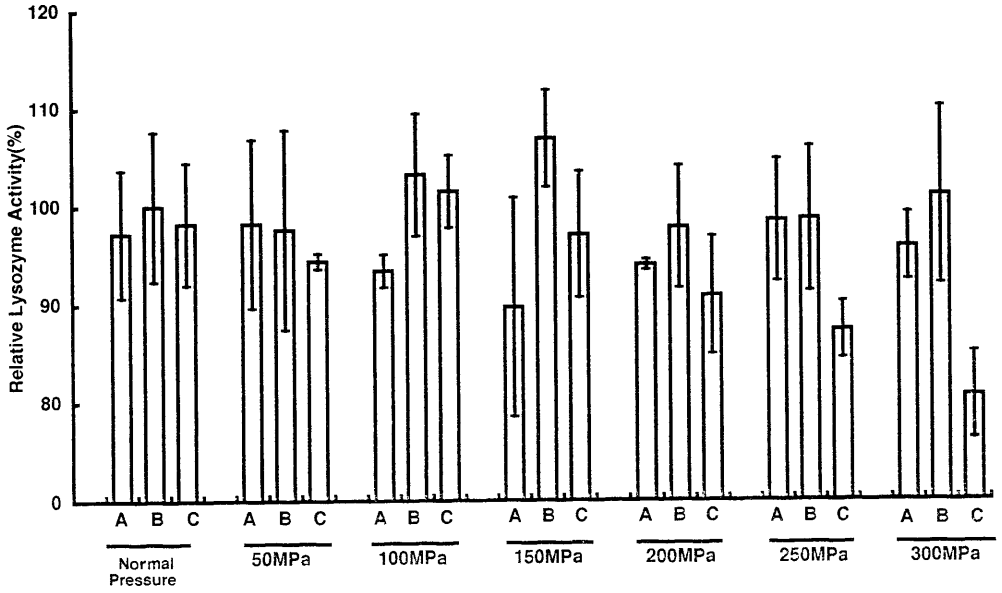


**Fig. 1.** Pressure tolerance of lysozyme. Relative lysozyme activities of lysozyme were determined using ML cell suspension after high pressure treatment over the pressure range of 50–300 MPa for 10 min at 0°C (A), 35°C (B as control) or 60°C (C). The relative lysozyme activity was expressed as percentage of lysozyme activity of high pressure treated lysozyme against non-treated lysozyme.

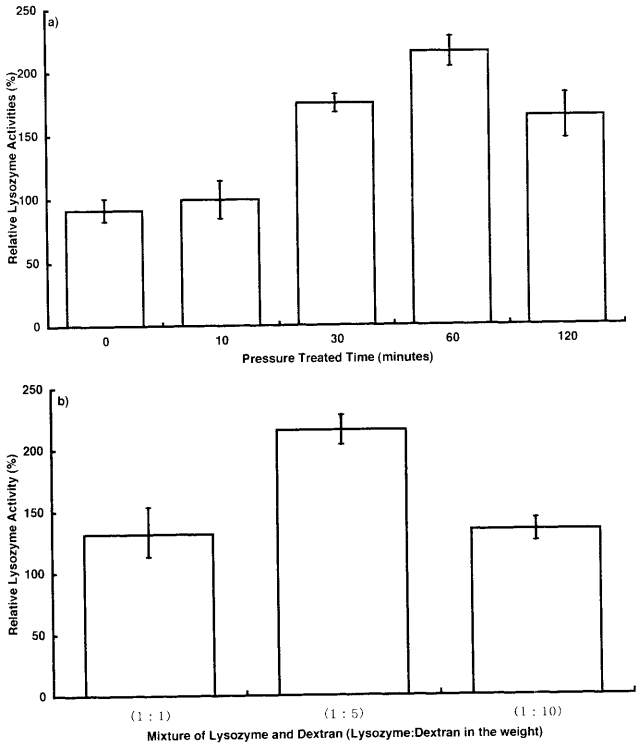


**Fig. 3.** Relative lysozyme activity of lysozyme-dextran complex. The relative lysozyme activity was expressed as percentage of lysozyme activity of high pressure treated lysozyme against non-treated lysozyme. a) Influence of treatment time (min): Lysozyme-dextran complexes were prepared from a mixture of lysozyme and dextran in the weight ratio of 1 : 5 by high pressure treatment over the pressure range of 150 MPa at 60°C for 10, 30, 60 and 120 min. b) Influence of mixture constitution in treated solution: Lysozyme-dextran complexes were prepared from a mixture of lysozyme and dextran in the weight ratio of 1 : 1, 1 : 5 and 1 : 10 by high pressure treatment over the pressure range of 150 MPa at 60°C for 60 min.

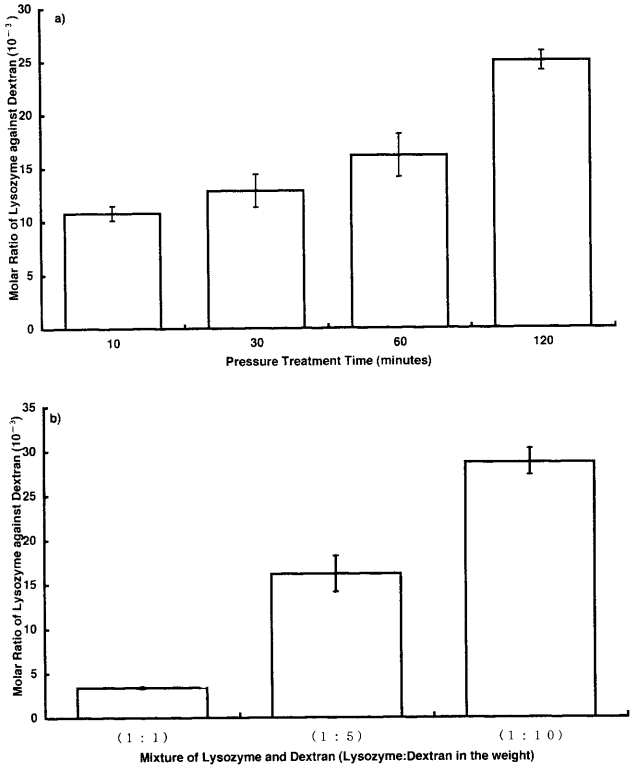


**Fig. 4.** Molar ratio of lysozyme against dextran in lysozyme-dextran complex. The molecular ratio of lysozyme to dextran was estimated from the amounts of protein and saccharide in the lysozyme-dextran complex determined by the Folin-Lowry method and phenol-sulfuric acid method. The molar ratio of lysozyme to dextran in the lysozyme-dextran complexes. a) Influence of treatment time (min): Lysozyme-dextran complexes were obtained by the same procedure as described in Fig. 3 a. b Influence of mixture constitution: Lysozyme-dextran complexes were obtained by the same procedure as described in Fig. 3 b.

the ML cell suspensions. The relative lysozyme activity of the lysozyme-dextran complex prepared from the premixed solution of lysozyme and dextran in the weight ratio of 1 : 5 (lysozyme : dextran) was increased with treatment time up to 60 min, but decreased at 120 min (Fig. 3a). Furthermore, the lysozyme-dextran complexes had higher relative lysozyme activities than the native lysozyme, and the lysozyme-dextran complex kept the highest relative lysozyme activity that was prepared by the high pressure treatment under the conditions of 150 MPa for 60 min at 60°C. The suitable weight ratio of lysozyme and dextran was thought to be 1 : 5 (lysozyme : dextran) (Fig. 3b).

*Binding ratio of lysozyme-dextran complex* Figure 4a shows the molar ratio of lysozyme to dextran in the lysozyme-dextran complexes, which were prepared from the lysozyme-dextran solutions in the weight ratio of 1 : 5 (lysozyme : dextran) by high pressure treatment at 150 MPa and 60°C. The high pressure treatment was carried out for 10, 30, 60 and 120 min, and the increasing tendency of lysozyme in the complex was also observed at about 11, 13, 16 and $25\times10^{-3}$ of lysozyme against dextran, respectively. These indicated that the molar ratio of lysozyme in the complex was increased with treatment time. The increasing tendency of the lysozyme in the complex was also obtained with an increase in the dextran content in the premixed solution, such as 3, 16 and $29\times10^{-3}$ of lysozyme against dextran by high pressure treatment at 150 MPa and 60°C from the mixture of lysozyme and dextran in the weight ratio of 1 : 1, 1 : 5 and 1 : 10 (lysozyme : dextran), respectively (Fig. 4b).

## Discussion

Lysozyme has a potent tolerance to high pressure treatment (Gekkou, 1994), though it is well known that the enzyme is denatured under the influence of pressure, temperature and treatment time during the high pressure treatment and its enzymatic activity is decreased (Frauenfelder *et al.*, 1987; Hara *et al.*, 1989). The lysozyme has four disulfide-bonds in its molecule (Funatsu & Tsuru, 1977). Kundrot and Richards reported that the entire molecule volume of the lysozyme was decreased only 0.47% at a pressure of 1,000 atm at low temperature (Kundrot & Richards, 1987). The disulfide-bonds contributed to the stabilization of the lysozyme against the high pressure treatments. In this study, the effects of high pressure treatments on the lysozyme were investigated by measuring the activity of the lysozyme which was treated at three levels of temperature (0°C, 35°C and 60°C) and seven levels of pressure (0, 50, 100, 150, 200, 250 and 300 MPa). At 0°C and 35°C, lysozyme had not denatured and perfectly kept

its activity during high pressure treatment of 300 MPa, however, its activity decreased with the increase in pressure levels at 60°C. This result suggested that the high pressure treatment at high temperature decreased the renaturation capacity of the lysozyme and the temperature played a more important role than the pressure level on the stabilization of the enzymatic activity (Kunugi & Tanaka, 1994).

The lysozyme-dextran complex was able to be produced by the Maillard reaction based on the method of Kato (Kato, 1994), though this method required a long time at normal pressure. Tamaoka *et al.* reported that the formation of melanoidin was suppressed by high pressure treatment (Tamaoka *et al.*, 1991). We examined the production of the lysozyme-dextran complex by the Maillard reaction under high pressure conditions. It was then considered that the use of the excessive high pressure destroyed the higher-order structure of the lysozyme, while lower pressure did not affect the structure, and the optimum pressure condition for the production of the lysozyme-dextran complex was desirable in order to produce the minimum irreversible denaturation on the lysozyme. From these hypotheses, it was postulated that the production of the lysozyme-dextran complex was desirable at a pressure of 150 MPa at 60°C. This high pressure level was the most favorable condition to maintain the lysozyme activity. The preparation of the lysozyme-dextran complex under the pressure of 150 MPa at 60°C increased the molar ratio of the lysozyme to dextran in the complex with increasing treatment time. For 120 min, however, the lysozyme activity of the complex decreased as compared with that of treatment for 60 min. The decrease in the relative lysozyme activity did not occur due to only high pressure, but by the exposure of the lysozyme for long time at high pressure and high temperature. It was indicated that the optimum time to produce the lysozyme-dextran complex at a pressure of 150 MPa at 60°C was 60 min. The difference in the relative lysozyme activity was observed in the lysozyme-dextran complex prepared from the solution with the different ratio of lysozyme and dextran. Upon treatment of the premixed solution for 60 min at 60°C at a pressure of 150 MPa, the molar ratio of lysozyme in the lysozyme-dextran complex increased with an increase in the dextran in the premixed solution containing lysozyme and dextran. On the other hand, the relative lysozyme activity in the lysozyme-dextran complex decreased with an increase in the dextran in the premixed solution. The lysozyme-dextran complex, which was prepared from the lysozyme-dextran mix in the weight ratio of 1 : 5 by the Maillard reaction was constituted in the molar ratio of 1 : 2 (Nakamura *et al.*, 1991), though the transformation of the higher-order structure of the lysozyme by binding dextran was prevented by the amino acids group in the rigid globular protein of the lysozyme (Kato, 1994). The increase in relative lysozyme activity was observed in the lysozyme-dextran complex prepared by high pressue treatment. However, Nakamura *et al.* reported that the relative lysozyme activity decreased in the complex by the Maillard reaction at normal pressure (Nakamura *et al.*, 1991). On the other hand, Fukui and Miyatani suggested that the increase in the relative lysozyme activity in the lysozyme-dextran complex resulted from amphotericity by complexation of the lysozyme and dextran, and the increase in affinity of the lysozyme-dextran complex to the cell wall (Fukui & Miyatani, 1978).

It was concluded that the increase in relative lysozyme activity in the lysozyme-dextran complex prepared by high pressure treatment was caused by the hydrophilic activity of a large amount of dextran in the complex by complexation of the lysozyme and dextran.

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