Enzymatic Properties of Rhea Lysozyme

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Rhea lysozyme was analyzed for its enzymatic properties both lytic and oligomer activities to reveal the structural and functional relationships of goose type lysozyme. Rhea lysozyme had the highest lytic activity at pH 6, followed by ostrich and goose at pH 5.5-6, whereas the optimum of cassowary was at pH 5. pH profile was correlated to the net charge of each molecule surface. On the other hand, the pH optimum for oligomer substrate was found to be pH 4, indicating the mechanism of rhea catalysis as a general acid. The time-course of the reaction was studied using β,1,4-linked oligosaccharide of N-acetylglucosamine (GlcNAc) with a polymerization degree of n (GlcNAc)n (n = 4, 5, and 6) as the substrate. This enzyme hydrolyzed (GlcNAc)n in an endo-splitting manner, which produced (GlcNAc)3 + (GlcNAc)5 predominating over that to (GlcNAc)3+ (GlcNAc)4. This indicates that the lysozyme hydrolyzed preferentially the third glycosidic linkage from the nonreducing end. Theoretical analysis has shown the highest rate constant value at 1.5 s⁻¹ with (GlcNAc)6. This confirmed six substrate binding subsites as goose lysozyme (Honda, Y., and Fukamizo, T., Biochim. Biophys. Acta, 1388, 53–65 (1998)). The different binding free energy values for subsites B, C, F, and G from goose lysozyme might responsible for the amino acid substitutions, Asn122Ser and Phe123Met, located at the subsite B.

Key words: rhea; G-type lysozyme; binding subsite; lysozyme

Lysozymes (EC 3.2.1.17) are self-defense enzymes found mainly in egg whites, tears, and various other secretions of eukaryotic cells. These enzymes are one of the most structurally well-characterized carbohydrate hydrolases by cleavage of the glycosidic linkage between N-acetyl muramic acid and GlcNAc in the bacterial cell walls. They are classified into three types: chicken type (C-type),1–3 phage type (T4-type),4,5 and goose type (G-type),6–8 based on the similarity of amino acid sequences. These three different classes of lysozymes have overall similarities in tertiary structure, although their amino acid sequences are almost entirely different. Abundant information on the structural properties, enzymatic mechanisms, and three-dimensional structures of C-type and T4-type lysozymes have been accumulated thus far, but information on G-type lysozyme is quite limited. The primary structure has been reported in 1980–2004 for only five G-type lysozymes, those from black swan,10 ostrich,11 embden goose,5 cassowary,12 and rhea egg white.13 Additionally, three G-type lysozyme genes from chicken,4 Japanese flounder,15 and orange-spotted grouper16 have been reported. Enzymatic mechanism information on G-type lysozyme is quite limited as well; only two three-dimensional structures for G-type lysozyme and only one form with oligosaccharide have been elucidated. The subsite structure of G-type lysozyme has been determined only for subsites B, C, and D with the crystal structure of a stable complex between goose lysozyme and trimer of GlcNAc.17 Additionally, Honda and Fukamizo reported the binding mode of GlcNAc oligomer to goose lysozyme, and postulated that goose lysozyme has six substrate binding subsites (B, C, D, E, F, and G).18 Therefore, the clarification of amino acids that are actually involved in substrate binding, especially at subsites E, F, and G, is of particular interest.

The active sites of this enzyme have a highly conserved Glu residue, which is believed to act as a general acid in catalysis (Glu35 in C-type lysozyme, Glu11 in T4-type lysozyme, and Glu73 in G-type lysozyme).17 On the other hand, the additional carboxylate ion of an Asp residue shows variability among the molecules: G-type lysozyme has no apparent counterpart to either Asp52 in C-type lysozyme or Asp20 in T4-type lysozyme.17 However, as yet, a definitive confirmation of the precise role of the second acidic residue in catalysis has not been obtained. G-type lysozyme also differs from the C-type in that it is much more specific for peptide-substituted substrates.20 C-type lysozyme hydrolyzes a homopolymer (chitin) effectively, while G-type lysozyme is a poor catalyst of the hydrolysis of this substrate. The differences in substrate specificity between these lysozymes are not well understood.

To overcome the lack of understanding of the
Materials and Methods

**Materials.** Rhea eggs were obtained from Izu Shaboten Park, Ito, Shizuoka, Japan. Ostrich, cassowary, and goose eggs were obtained from the Kumamoto City Zoological and Botanical Gardens, and the Kumamoto and Hirakawa Zoos, Kagoshima, Japan. Chemical reagents were all of analytical grade and commercially available. Enzyme purification was done by the methods reported previously.\(^{13}\)

**Lytic activity measurement.** Lytic activity was assayed using the lyophilized cell wall of *Micrococcus luteus* (M-3770, Sigma) as a substrate.

For analyzing the enzymatic properties, the substrate was prepared in 0.1 M Britton Robinson buffer, pH 2–10, at a range of 4.0–7.0 with 0.5 unit intervals of increasing pH level. For the effects of pH on lytic activity, 0.1 M Britton Robinson buffer, pH 6.0, for pH stability on lytic activity, 0.1 M Britton Robinson buffer, pH 5.0, for thermal stability on lytic activity, and 0.1 M phosphate buffer, pH 6.0, for optimum temperature on lytic activity were used. Sample solutions (100 μl) were added to 3 ml of the substrate suspension in the designated buffer, adjusted to OD 1.0 at 540 nm. The activity unit was evaluated as the decrease of absorbance at 540 nm, 25 °C. One enzyme unit was defined as the amount causing a decrease of 0.1 absorbance units at 540 nm (A540) in the reaction for 1 min.

**Effects of pH on lytic activity.** The enzyme dissolved in distilled water (0.067 M) was added to substrate solution (pH 2.0–10.0) of 0.1 M Britton Robinson buffer. The activity was then measured at 25 °C for 5 min.

**pH stability on lytic activity.** The enzyme was dissolved in various pHs of 0.1 M Britton Robinson buffer from pH 2.0 to 10.0 to a final concentration of 0.067 μM. Then the enzyme solution was incubated at 37 °C for 1 h, 2 h, and 4 h. The residual activity was measured as described in “Lytic activity measurement” in 0.1 M Britton Robinson Buffer, pH 6.0. The pH stability of lytic activity as a function of temperature (50 °C and 65 °C) was also examined. The enzyme was dissolved in various pHs of 0.1 M Britton Robinson buffer (pH 2.0–10.0) and then incubated at 50 °C and 65 °C for 1 h. Then the residual activity was measured in the manner described above.

**Optimum temperature on lytic activity.** The optimum temperature for lytic activity was assayed at temperatures ranging from 20 °C to 80 °C. The substrate solution was dissolved in 0.1 M phosphate buffer, pH 6.0, and incubated at various temperatures (20 °C–80 °C) for 10 min. The enzyme dissolved in distilled water (0.067 μM) was added and then the activity was measured at designated temperature for 1 min.

**Thermal stability on lytic activity.** The enzyme was dissolved in 0.1 M Britton Robinson Buffer pH 5.0 to a final concentration of 0.067 μM and then incubated at various temperatures from 20 °C to 80 °C for 10 min, 30 min, and 60 min. Then the activity was measured as described in “Lytic activity measurement” in 0.1 M phosphate buffer, pH 5.0.

The thermal stability of lytic activity at high temperature was also examined. The enzyme was dissolved in 0.1 M Britton Robinson buffer, pH 5.0, to a final concentration of 0.2 μM and incubated at 90 °C for 1 to 5 h. Then the residual activity was measured in the manner described above.

**Oligomer activity measurement.** For a pH activity profile of the rhea lysozyme, the optimal pH was measured by the method of Masaki,\(^{21}\) with a slight modification. Namely, the reaction mixture containing 0.1 mM lysozyme and 1 mM (GlcNAc)\(_5\) was incubated at 50 °C for 40 min in various pHs (pH 2.0–7.0) using the following buffer: 0.01 M sodium acetate buffer for pH 2.0 to 5.0, and 0.01 M phosphate buffer for pH 6 and 7. (GlcNAc)\(_5\) was prepared by acid hydrolysis of chitin followed by charcoal celite column chromatography.\(^{22}\) After 40 min, 200 μl of the reaction mixture was withdrawn and rapidly chilled in a Kool Kup (Towa, Japan). The reaction mixture was centrifuged with Ultrafree C3LCC (Millipore, U.S.A.) for 1 h at 7,000 rpm and 4 °C, then 90 μl of distilled water was added and centrifuged again, and this step was repeated 2 times. The filtrate was lyophilized. The dried sample was dissolved in 50 μl of ice-cold water, and 10 μl of the solution was applied on TSK gel G-Oligo-PW column (7.8 × 600 mm, Tosoh, Japan) using a Jasco 800 series HPLC. Elution was done with distilled water at room temperature at a flow rate of 0.3 ml/min. Each (GlcNAc)\(_n\) concentration was calculated from the peak area monitored by ultraviolet absorption at 220 nm, using the standard curve obtained from authentic saccharide solutions.

**Enzyme action on N-acetylglucosamine oligosaccharides (GlcNAc)\(_{4, 5}\) and \(_6\) substrate.** Rhea lysozyme activity was measured as described in “Oligomer activity measurement” in 0.01 M acetate buffer, pH 4.0 (optimal pH), with a given reaction time. The relative error was defined as \((y – x)/x \times 100\), where \(x\) is the concentration of initial substrate and \(y\) is the recovered concentration of all (GlcNAc)\(_n\) in (GlcNAc)\(_1\) units.

The rate equation of the lysozyme-catalyzed reaction on the initial substrate (GlcNAc)\(_5\) was numerically...
solved to obtain the calculated time–courses. A kinetic model of the lysozyme-catalyzed reaction of (GlcNAc)$_n$ has been reported, and is schematically presented in Fig. 1. In the calculation, the rate constants were solved repeatedly while changing the values of the binding free energies or the rate constants, $k_{+1}$, $k_{-1}$, and $k_{+2}$, so that the calculated time–courses fitted those experimentally obtained.

Results and Discussion

As previously reported, the primary structure of novel G-type lysozyme from rhea egg white has been established. This enzyme, composed of 185 amino acid residues, has been shown to have the highest homology to that of ostrich lysozyme and also a similar amino acid composition to those of black swan, goose, and cassowary lysozymes. The six substituted positions were newly found at positions 3 (Asn), 9 (Ser), 43 (Arg), 114 (Ile), 127 (Met), and 129 (Arg) when compared with those of other G-type lysozymes. Interestingly, rhea lysozyme carries amino acid substitutions at the binding site compared with goose and black swan lysozyme (Asn122Ser and Phe123Met) (Fig. 2). To elucidate the structural and functional relationship, we analyzed the enzymatic properties of both lytic and oligomer activities.

Effects of pH on lytic activity of rhea lysozyme

The lytic activity of rhea lysozyme against M. luteus...
was examined at various pHs (pH 2.0–10.0) using 0.1 M Britton Robinson buffer as shown in Fig. 3. Rhea had the highest lytic activity at pH 6, followed by ostrich and goose at pH 5.5–6, whereas the optimum of cassowary was at pH 5. Rhea had higher activity than those of the other three G-type lysozymes at optimum pH. No G-type lysozymes had activity on the acidic (below pH 3.0) or alkaline (upper pH 8.0) sides.

According to the lytic mechanism of chicken lysozyme (C-type lysozyme), the activity is accomplished by an electrostatic interaction between the positive charge (net charge) of the lysozyme and the negative charge of the bacterial cell wall. In fact, we found clear evidence that the optimum pH of these enzyme groups correlated to the net charge of the molecule. It was observed that rhea lysozyme had the highest positive net charge, of +17, followed by ostrich and goose with +16 and cassowary with +15 (Table 1). The amino acids on the surface of the protein affect the net charge of the protein molecule that plays an important role in the electrostatic interaction, indicating that the positive charge (net charge) of the lysozyme and the negative charge of the cells is responsible for the lytic activity of the lysozyme.

Regarding the pH stability for lytic activity, the rhea and ostrich lysozymes showed a wide stable range (pH 3.0–10.0) in 0.1 M Britton Robinson buffer at 37°C up to 4 h for lytic activity (Fig. 4). This result was the same as for cassowary, which had slightly lower lytic activity on the alkaline side. Basically, rhea and ostrich lysozymes were stable, but on the alkaline side (pH 7.0–10.0) the activity showed lower on the alkaline side (pH 7.0–10.0) than on the acidic side. To clarify the

Table 1. Amino Acid Compositions of Rhea and Other Goose Type Lysozymes

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Rhea</th>
<th>Ostrich</th>
<th>Goose</th>
<th>Cassowary</th>
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| Total       | 185  | 185     | 185   | 185       |
| Net Charge  | +17  | +16     | +16   | +15       |

Fig. 3. Effects of pH on the Lytic Activity of Rhea, Ostrich, Goose, and Cassowary Lysozymes against *M. luteus*.

The enzyme dissolved in distilled water (0.067μM) was added to substrate solution (pH 2.0–10.0) of 0.1 M Britton Robinson buffer, at a range of 4.0–7.0 with 0.5 unit intervals of increasing pH level. The activity was then measured at 25°C for 5 min. Closed triangle, closed circle, opened circle, and closed square indicate rhea, ostrich, goose, and cassowary lysozymes respectively.
instability on the alkaline side, we examined the pH stability of this enzyme at elevated temperatures of 50°C and 65°C to emphasize the effect of pH (Fig. 5). At 50°C and 65°C, the activities were markedly decreased on alkaline side.

**Effects of temperature and thermal stability on lytic activity of rhea lysozyme**

The optimum temperature for lytic activity of rhea and ostrich lysozymes was assayed in the range of 20°C to 80°C in 0.1 M phosphate buffer, pH 6.0 (Fig. 6). Both lysozymes had an optimum temperature at 30–40°C, as previously reported for cassowary lysozyme.27)

The thermal stability of lytic activity was also investigated at temperatures from 20°C to 80°C in the same buffer (Fig. 7). Both rhea and ostrich lysozymes had thermal stability up to 40°C for 30 min. For 60 min treatment, both lysozymes gradually lost their activity with the same pattern as cassowary lysozyme.27) Another notable feature was found for thermal stability after treatment at high temperature (80°C). 20% of remaining activity was found in this condition. This marked stability at high temperature was also proved by the incubation of this enzyme at 90°C up to 5 h. The activities of rhea and ostrich lysozymes were kept at 40% for 1 h and gradually decreased during incubation, but 10% of activity remained after 5 h incubation, as shown in Fig. 8. This thermal stability is perhaps caused by the hydrophobic core structure composed of the three α-helices in spite of considerable amino acid substitutions in each enzyme.12)

**Effects of pH for oligomer substrate of rhea lysozyme**

The pH dependence by oligomer study on the substrate binding site was measured using (GlcNAc)₅ as a substrate in various pHs (pH 2.0–7.0). The rate of hydrolysis of (GlcNAc)₅ was most rapid at pH 4 (Fig. 9). Between pH 3.5 and 4.0, rhea lysozyme was optimally active and its activity dropped sharply below pH 3 and above pH 5. This suggests that the rapid decrease on either side of this optimal pH is characteristic of an ionizable catalytic amino acid. From the reported lysozyme mechanism, the active sites of this enzyme have a highly conserved Glu residue, which is
believed to act as a general acid in catalysis as proton donors (Glu35 in C-type lysozyme, Glu11 in T4-type lysozyme, and Glu73 in G-type lysozyme).

Therefore, at pH 4, the pH optimum for the hydrolysis of (GlcNAc)$_3$ by rhea lysozyme, Glu73 is in the unionized COOH form. On the other hand, the additional carboxylate ion of an Asp residue shows variability among the molecules: G-type lysozyme has no apparent counterpart to either Asp52 in C-type lysozyme or Asp20 in T4-type lysozyme. However, as yet, a definitive confirmation of the precise role of the second acidic residue in catalysis has not been obtained. Therefore the mechanism of rhea catalysis was originally proposed because it acts as a general acid to protonate the leaving group and/or a base to stabilize the transition state.
The optimum pH of rhea lysozyme activity for polymer substrate and for oligomer substrate has shown different values (pH 6 and pH 4). These studies suggest that the rhea catalysis mechanism using polymer substrate was correlated to the net charge of the protein molecule, while the oligomer substrates that bind to the catalysis site were independent of the net charge of the protein molecule.

**Activity of rhea lysozyme against N-acetylglucosamine oligosaccharides (GlcNAc)n**

The lysozyme-catalyzed reactions were effectively investigated using the experimental time–course of oligosaccharide digestion, because much information about lysozyme catalysis, such as the splitting mode, subsite structure, and transglycosylation, can then be obtained. However, the mode of enzyme action of G-type lysozyme toward (GlcNAc)n has been examined only for goose lysozyme. Therefore, the experimental time–course of (GlcNAc)n digestion by rhea lysozyme was analyzed not only to obtain the essential basic information on G-type lysozyme but also to elucidate the effects of amino acid substitutions on the subsites binding mode.

The experimental time–course of rhea lysozyme for (GlcNAc)4, 5, and 6 were analyzed and are shown in Fig. 10. Under the experimental conditions used, (GlcNAc)6 was hydrolyzed rapidly and almost completely after 40 min of reaction (Fig. 10A). Rhea lysozyme produced abundant amounts of (GlcNAc)3 and lesser amounts of (GlcNAc)2 and 4, while (GlcNAc)1 and 5 were produced in much smaller amounts. This indicates that rhea lysozyme hydrolyzed the substrate in an endo-splitting manner. Considering the fact that two molecules of (GlcNAc)3 are produced from (GlcNAc)6, the frequency of cleavage to (GlcNAc)3 + (GlcNAc)1 appears to be considerably higher than that of cleavage to (GlcNAc)3 + (GlcNAc)4. Thus rhea lysozyme appears preferentially to hydrolyze the third glycosidic linkage from the nonreducing end of (GlcNAc)6. Oligosaccharide products with a degree of polymerization higher than that of the initial substrate were not observed, indicating that rhea lysozyme does not catalyze transglycosylation.

The substrate (GlcNAc)5 was then digested with rhea lysozyme and the time–course was obtained under the same conditions (Fig. 10B). The substrate was hydrolyzed mainly to (GlcNAc)2 + (GlcNAc)3 with much less cleavage to (GlcNAc)1 + (GlcNAc)4. Rhea lysozyme also hydrolyzed (GlcNAc)5 in an endo-splitting manner, although the reaction rate was lower than that of (GlcNAc)6 hydrolysis.

For further information on the splitting mode of rhea lysozyme hydrolysis, we obtained the time–course of (GlcNAc)4. The order of the amounts of the products was (GlcNAc)2 > (GlcNAc)1 = (GlcNAc)3. The reaction rate was much lower than those of (GlcNAc)5 and (GlcNAc)6 hydrolysis. After a long incubation time of 12 h, large amounts of initial substrate remained (Fig. 10C).

As the result, the product distribution produced by rhea lysozyme for (GlcNAc)6 was similar to that of goose lysozyme. These two lysozymes are believed to share the same type of subsite structure.

However, the profile of the time–course of (GlcNAc)6 degradation for rhea lysozyme differed slightly from that of goose lysozyme. Intermediate substrate (GlcNAc)4 found in the reaction of (GlcNAc)6 was gradually decomposed compared with goose lysozyme, accompanied by a slight increase in the amount of (GlcNAc)1, (GlcNAc)2, and (GlcNAc)3 (Fig. 10A). The intermediate (GlcNAc)4 was probably hydrolyzed slowly and continuously to (GlcNAc)2 + (GlcNAc)3 or (GlcNAc)1 + (GlcNAc)3, which coincides with the product distribution on the time–course of (GlcNAc)4 (Fig. 10C). Further, (GlcNAc)4 on the time–course of (GlcNAc)3 hydrolysis also decomposed slowly (Fig. 10B). But this decomposition of the intermediate product (GlcNAc)3 was not observed in the time–course of goose lysozyme, suggesting that the binding mode of the oligosaccharide for rhea is similar to that of goose lysozyme, but somewhat different in some details.

**Theoretical analysis of the experimental time–course**

To evaluate the rhea lysozyme-catalyzed reactions, the experimental time–course of (GlcNAc)6 degradation was analyzed by computer simulation in order to obtain the rate constants and the binding free energy values of the individual subsites. The theoretical model used for the analysis of goose lysozyme, in which six binding subsites (B, C, D, E, F, and G) and bond cleavage between subsite D and E were postulated, was employed in the analysis of the rhea lysozyme-catalyzed reaction. The calculated time–course of rhea lysozyme is shown in Fig. 10A and the reaction parameters obtained are summarized in Table 2. We obtained optimized free energy change values of −0.55, −1.6, +4.2, −1.5, −2.1, and −2.6 kcal/mol for the corresponding subsites of rhea lysozyme. The rate constant k for hydration was set at the value estimated for goose lysozyme, 100.0 s⁻¹. The rate constants k1 for the cleavage of the glycosidic linkage were estimated to be 0.5 s⁻¹ for (GlcNAc)4, 1.2 s⁻¹ for (GlcNAc)5, and 1.5 s⁻¹ for (GlcNAc)6. (GlcNAc)6 has the highest rate constant value, suggesting that rhea lysozyme has six substrate binding subsites similar to that deduced for goose lysozyme.

Although the subsite structure of rhea lysozyme was estimated to be identical to that of goose lysozyme, rhea had different binding free energies at subsites B, C, F, G than that of goose lysozyme. The binding free energy values for subsite B for rhea lysozyme increased by 0.05 kcal/mol, while for subsites C, F, and G they decreased by 0.6, 0.5, and 0.2 kcal/mol, respectively compared with those of goose lysozyme (Table 2). Based on the structural determination of the amino acid
substitutions found in rhea lysozyme, Asn122Ser and Phe123Met are located at binding subsite B comparing goose lysozyme. These data indicate that the substrate binding mode of rhea lysozyme is different from that of goose lysozyme at or near subsites B, C, F, and G, and that the characteristic time-course of rhea lysozyme might be due to the change of the affinities for the substrate at these sites. Further, in the three-dimensional structure of goose lysozyme, subsites E, F, and G were composed of some part of the N-terminal region. The possible chains of amino acid residues 73–86 (coil region and β1), 32–43 (h2 and h3), and 18–25 (h1) might contact to sugar rings at subsites E, F, and G respectively (Fig. 2). Rhea has a large number of amino acid substitutions in the N-terminal part as compared with goose lysozyme, suggesting that these substitutions might affect the binding affinity of the substrate at subsites F and G of rhea lysozyme. The rate constant

Fig. 10. The Time–Courses of \((\text{GlcNAc})^n\) Catalyzed by Rhea Lysozyme.

The initial concentrations of enzyme and substrate were 0.1 and 1 mM respectively. Relative error indicated the recovery of the observed value at each reaction time calculated, as described in “Materials and Methods”. A and B show both the experimental time–courses and the calculated time–courses of \((\text{GlcNAc})_n\) and \((\text{GlcNAc})_5\) respectively. C shows only the experimental time–courses of \((\text{GlcNAc})_5\). Symbols represent the experimental points for \((\text{GlcNAc})_1\) (open circle), \((\text{GlcNAc})_2\) (open square), \((\text{GlcNAc})_3\) (closed triangle), \((\text{GlcNAc})_4\) (open triangle), \((\text{GlcNAc})_5\) (closed circle), and \((\text{GlcNAc})_6\) (closed square). For A and B, solid lines indicate the theoretical time–course calculated with the binding free energy values listed in Table 2. Numerals in the figures are the polymerization degrees of the reaction product species.
value $k_1$ for rhea lysozyme was much larger than that for goose lysozyme (Table 2), but a reasonable explanation for this cannot be given at present because of the differences in the experimental conditions used.

Further to confirm the validity of the parameter values obtained, we tried to calculate the time–course of (GlcNAc)$_6$ degradation using the values listed in Table 2, together with the value for goose lysozyme. As shown in Fig. 10B, the profile of the theoretical time–course was in good agreement with that obtained experimentally. Therefore we conclude that the kinetic parameters estimated from (GlcNAc)$_6$ hydrolysis are most appropriate for the rhea lysozyme-catalyzed reaction. However, the binding free energy at subsite B for (GlcNAc)$_6$ was lower than that of (GlcNAc)$_3$ by −0.25 kcal/mol, indicating the difference in the occupation of subsites by the substrate.

In conclusion, we found new enzymatic properties based on the structure of rhea lysozyme. On the basis of a lytic activity study, we found that G type lysozyme showed a pH profile correlated to the net charge of each molecule. Rhea lysozyme had the highest lytic activity at pH 6, followed by ostrich and goose at pH 5.5–6, whereas the optimum of cassowary was at pH 5, accompanied by a positive net charge of +17, +16, and +15 respectively. On the other hand, as for pH dependence by the oligomer study it was found that the optimum pH for lysozyme catalysis is pH 4, indicating that the mechanism of rhea catalysis is a general acid to protonate the leaving group and/or a base to stabilize the transition state independent of net change of protein molecule.

G-type lysozymes have conserved amino acid sequences at the regions of h5, h7, and h8 that compose the hydrophobic core in the molecule as well as the conservation of the acidic catalytic residue, Glu73. Rhea lysozyme shares 83% amino acid identity with goose lysozyme, and the substrate-binding residues proposed for goose lysozyme$^{18}$ are mostly conserved in rhea lysozyme, but amino acid residues at substrate binding subsite B have been found as substitutes (Asn122Ser and Phe123Met). To elucidate the enzyme action and catalytic mechanism, rhea lysozyme was estimated by oligomer study. Since the binding free energy values at subsite B are different between rhea and goose lysozyme (Table 2), the different cleavage profiles of the two lysozymes can be partly explained by these two substitutions. The binding energy changed at subsite C observed for rhea lysozyme might be due to the effect of conformational change on subsite B. The difference in free energy change values at subsites F and G might also contribute to the different cleavage profiles. Further discussion, however, may be provided after establishment of the structure of subsites F and G.

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