The Primary Structure of Cassowary (Casuarius casuarius) Goose Type Lysozyme

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The complete amino acid sequence of cassowary (Casuarius casuarius) goose type lysozyme was analyzed by direct protein sequencing of peptides obtained by cleavage with trypsin, V8 protease, chymotrypsin, lysyl endopeptidase, and cyanogen bromide. The N-terminal residue of the enzyme was deduced to be a pyroglutamate group by analysis with a LC/MS/MS system equipped with the oMALDI ionization source, and then confirmed by a glutamate aminopeptidase enzyme. The blocked N-terminal is the first reported in this enzyme group. The positions of disulfide bonds in this enzyme were chemically identified as Cys4-Cys60 and Cys18-Cys29. Cassowary lysozyme was proved to consist of 185 amino acid residues and had a molecular mass of 20408 Da calculated from the amino acid sequence. The amino acid sequence of cassowary lysozyme compared to that of reported G-type lysozymes had identities of 90, 83, and 81%, for ostrich, goose, and black swan lysozymes, respectively. The amino acid substitutions at PyroGlu1, Glu19, Gly40, Asp82, Thr102, Thr156, and Asn167 were newly detected in this enzyme group. The substituted amino acids that might contribute to substrate binding were found at subsite B (Asn122Ser, Phe123Met). The amino acid sequences that formed three α-helices and three β-sheets were completely conserved. The disulfide bond locations and catalytic amino acid were also strictly conserved. The conservation of the three α-helices structures and the location of disulfide bonds were considered to be important for the formation of the hydrophobic core structure of the catalytic site and for maintaining a similar three-dimensional structure in this enzyme group.

Key words: cassowary; G-type lysozyme; amino acid sequence; lysozyme; disulfide bond

Lysozymes (EC 3.2.1.17) are considered to be self-defense enzymes, which are produced in many tissues of eukaryotic cells. They hydrolyze β-1,4-glycosidic linkages of peptidoglycans that are found in bacterial cell walls. Three distinct forms of lysozyme were typified by their amino acid sequences and tertiary structures, namely, chicken type (C-type),1–3) phage type (T4-type),4,5) and goose type (G-type).6–8) Among this enzyme group, in spite of the apparent lack of primary sequence correspondence, the three-dimensional structure of chicken lysozyme, T4 lysozyme, and goose lysozyme have been shown to share common tertiary structure elements.9–11) The catalytic mechanism of this enzyme group was proposed to follow the general acid catalysis involving the two acidic residues, the general acid-base glutamate, and the nucleophile aspartic acid residue.12) Lysozymes have highly conserved proton donors, Glu residues (Glu35, Glu11, and Glu73 in C-type, T4-type, and G-type lysozymes, respectively).3,5,7,10,11) On the other hand, one more carboxylate ion of the Asp residue that stabilizes the oxocarbonium ion of the reaction intermediate shows variability on their molecule. Recently, Weaver et al. showed the refined structure of goose lysozyme with a bound trisaccharide at subsites B, C, and D and proved that Glu73 of goose lysozyme corresponds to Glu35 of chicken lysozyme (and Glu11 of T4 lysozyme). Furthermore, they showed that the goose lysozyme lacks a catalytic Asp residue, a counterpart of Asp52 of chicken lysozyme.
(or Asp20 in T4 lysozyme). This information generates more questions regarding the function of Asp residues in enzymatic mechanism in this enzyme group. Monzinger et al. showed that the hydrolytic enzymes, namely chitosanases, chitinases, and lysozymes, have a structurally invariant core consisting of two helices and a three-stranded β-sheet which form the substrate binding site and catalytic cleft, in spite of the differences in their amino acid sequences, by the comparison of tertiary structure. Further, on the basis of structural criteria, these hydrolysases in the superfamily were divided into a procaryotic family (chitosanase and T4-type lysozyme) and an eucaryotic family (chitinase and G-type lysozyme). Honda and Fukamizo reported the binding mode of chitin oligomer to goose lysozyme and compared it with barley chitinase. The enzymatic hydrolysis of hexasaccharide was monitored by HPLC, and the reaction course was analyzed by a mathematical model in which six binding subsites (B, C, D, E, F, and G) and bond cleavage between sites D and E are postulated. The subsite structures of both enzymes proved to be identical and coincided with similarities in the tertiary structure of these enzymes. For the binding free energy change of the six binding subsites, B, C, D, E, F, and G, they found a major difference in kinetic parameters between the two enzymes lying in free energy value at site C and the catalytic potency of the glutamic acid residue. However from the study of free energy change of oligosaccharide hydrolysis by Honda and Fukamizo, six binding subsites (B, C, D, E, F, and G) of goose lysozyme were postulated. Therefore the clarification of which amino acids are involved in the binding sites especially in sites E, F, and G as well as specific amino acids for maintaining the structure of substrate binding cleft in three-dimensional structure of G-type lysozyme are of particular interest. To understand the structural basis of the functional diversity of this enzyme, we separated G-type lysozyme from cassowary (Casuarius casuarius) egg white. In this paper, we describe the complete amino acid sequence of the fourth G-type lysozyme, cassowary (Casuarius casuarius) lysozyme, as a mature protein along with the location of its disulfide bonds.

**Materials and Methods**

**Materials.** The cassowary lysozyme (Casuarius casuarius) was purified by the method reported in our previous paper. Cassowary eggs were obtained from Osaka Tennoji Zoo, Tennoji, Osaka and Kobe Oji Zoo, Nada, Kobe. All chemical reagents were analytical grade commercially available.

**Carboxymethylation and pyridylethylation.** Casso- ray lysozyme was reduced and carboxymethylated for structural analysis, according to the method of Crestfield et al., with slight modification. Namely, 10 mg of cassowary lysozyme was used and the Cm-lysozyme was purified through a Sephadex G-50 column (1.7 × 46 cm) in 0.2 M NH4OH. The Cm-lysozyme fraction was lyophilized.

Cassowary lysozyme was also reduced and pyridylethylated by the method of Cavins and Friedman. Ten mg of cassowary lysozyme was used and the Pe-lysozyme was purified through a Sephadex G-50 column (1.7 × 46 cm) in 5% formic acid. The Pe-lysozyme was lyophilized.

**Enzymatic digestion and chemical fragmentation.** Five mg of Pe-lysozyme was suspended in 1 ml of water and then digested with trypsin (1/50, w/w, TPCK, Cooper Biomedical Co., Japan) at 37°C, pH 8.0, for 4 h. Pe-lysozyme (5 mg) was digested with lysyl endopeptidase (1/250, w/w, Wako Pure Chemical Industries Ltd., Japan) in 0.01 M Tris-HCl buffer, pH 9.0, at 30°C, for 6 h. Five mg of Cm-lysozyme was digested with V8 protease (1/50, w/w, Boehringer Mannheim Co., Germany) in 50 mM potassium phosphate buffer, pH 7.8, at 37°C, for 10 h. Cm-lysozyme (5 mg) was also digested with α-chymotrypsin (1/25, w/w, Sigma Chemical Co., USA) at 37°C, pH 8.0, for 30 min. Chemical fragmentation was done with CNBr. One mg of Cm-lysozyme was dissolved in 700 μl of conc. HCOOH and then an additional 300 μl of water was added to make 70% HCOOH. Then 312 μl of 70% HCOOH containing a 200-fold molar excess of CNBr against Met residues in the molecule of cassowary lysozyme was added to the reaction mixture. The reaction mixture was kept at 4°C, for 24 h. After the reaction, 20 ml of water
was added and the mixture was evaporated to a final volume of 10 ml, three times. The CNBr fragments solution was then further purified by RP-HPLC.

**Peptide separation.** The digested peptides of cassowary lysozyme were purified by RP-HPLC column (YMC ODS 120A S-5, 4.6 × 250 mm, Yamamura Chemical Co., Japan) using a JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The peptides were developed with a gradient elution system of 0.1% TFA (solv. A), and 60% acetonitrile in solv. A (solv. B) (Solvent system 1). A gradient of 0–60% of solv. B in 150 min was made. The peptide was measured at 220 nm. Some peaks were rechromatographed by a gradient system of 5 mM phosphate buffer, pH 6.0 (solv. A), and 60% acetonitrile in solv. A (solv. B) (Solvent system 2). CNBr fragments were separated using the C4 column (C4, 4.6 × 250 mm, Bio-Rad Co., USA). The peptide fragments were developed with a gradient of Solvent system 1.

**Amino acid analysis and sequence analysis.** Peptides were hydrolyzed in evacuated sealed tubes with constant boiling HCl containing 0.05% β-mercaptoethanol at 110°C, for 20 h. Then the hydrolysates were analyzed with an amino acid analyzer (Model L-8500A, Hitachi Co., Japan). Tryptophan residues in peptides were calculated as previously reported. 36) Peptide sequencing was done by a DABITC/PITC double coupling manual micro sequencing method or a protein sequencer (abi Model 477A, Applied Biosystems Co., Japan, and PSQ-1, Shimadzu Co., Japan).

**Analysis of N-terminal amino acid sequence.** The N-terminal amino acid sequence of cassowary lysozyme was analyzed with tryptic peptides on an Applied Biosystems/MDS Sciex API QSTAR Pulsar Hybrid LC/MS/MS System equipped with oMALDI ionization source (MDS Sciex, API-QSTAR, Applied Biosystems, Toronto). The N-terminal amino acid blocking group of cassowary lysozyme was confirmed by the digested N-terminal tryptic peptide using PGAP. Digestion was done in 50 µl of 0.1 M phosphate buffer, pH 8.0, containing 5 mM dithiothreitol, 10 mM EDTA, and 5% glycerol with 1/20 (w/w, Boehringer Mannheim Co., Germany) PGAP at room temperature for 12 h, followed by further digestion with 1/20 (w/w) enzyme under the same conditions. The digested solution was purified by a RP-HPLC column (YMC ODS 120A S-5, 4.6 × 250 mm, Yamamura Chemical Co., Japan) using a JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The deblocked tryptic peptide was purified with a gradient elution of the Solvent system 1.

**Location of the disulfide bond.**

1. **Enzymatic digestion.** Ten mg of cassowary lysozyme dissolved in 0.5 ml of distilled water was adjusted to pH 1.8 with 1 M HCl and then digested with 0.2 mg (1/5, w/w) of acid protease (aspergillus acid protease type XIII, Sigma Chemical Co., USA). After digestion for 3 h, at 37°C, the pH was brought to 6.5 by addition of 0.5 M NaOH, and then further digested with 0.2 mg of thermolysin (1/5, w/w thermophilic-bacterial proteinase type X, Sigma Chemical Co., USA) in the presence of 0.001 M CaCl2 at 37°C, for 6 h. 37)

2. **Peptide separation.** The resulting enzymatic digest was put on a RP-HPLC column (YMC ODS 120A S-5, 4.6 × 250 nm, Yamamura Chemical Co., Japan). The peptides were developed with a gradient of the Solvent system 2. Disulfide bond (S-S) containing fractions were further purified on the same column, using a gradient of the Solvent system 1. Perforim oxidized peptides were separated on the same column, using the gradient of the Solvent system 1.

3. **Detection of cystine-containing peptides.** Cystine-containing peptides were detected using the method of Sueyoshi et al. 38) A sample of each fraction of HPLC elution (1/20 volume of each fraction; ~ 1–5 nmol each) was dried in a small test tube, in vacuo. Then 1.05 ml of 1 M borate buffer, pH 9.5 containing 4 mM EDTA, 75 µl of SBD-F solution (0.4 mg SBD-F per ml of distilled water), 15 µl of tributylphosphine, and 1.5 ml of dimethylacetamide solution (0.2 g of dimethylacetamide per ml of distilled water) were added. The reaction mixture was incubated at 60°C, for 1 h. After cooling to room temperature, the fluorescence intensity of the reaction mixture was measured using 1 nmol of GSSG as an external standard at 385 nm for excitation and 515 nm for emission by a fluorescence spectrophotometer (Model F4500, Hitachi Co., Japan).

4. **Performic acid oxidation.** S-S containing peptide fractions were dried in small test tubes. The peptides were oxidized with 100 µl of performic acid, prepared by the incubation of 950 µl of formic acid with 50 µl of 30% hydrogen peroxide at 25°C, for 2 h. After the reaction of 30 min at −10°C, the peptides were dried under a vacuum and dissolved with a small volume of 0.1% TFA and then separated on RP-HPLC (YMC ODS 120A S-5, 4.6 × 250 mm, Yamamura Chemical Co., Japan).

**Results and Discussion**

**Complete amino acid sequence of cassowary lysozyme**

As previously reported, this lysozyme has been simply purified by the method composed of pH treatments and cation exchange column chromatographies. The molecular mass was around 20800 Da by SDS-PAGE. This coincided with those of the reported G-type lysozymes. Further the amino acid composition of this lysozyme was shown to have the
greatest similarity to that of ostrich lysozyme and also had similarities to those of black swan and goose. To confirm it to be the G-type lysozyme, the N-terminal amino acids of this protein were sequenced. However no N-terminal was detected.\textsuperscript{33)}

The strategy used to determine the complete amino acid sequence of cassowary lysozyme is summarized in Fig. 1. The amino acid sequences inside of the polypeptide chain were determined on tryptic peptides and then overlapped by V8 protease peptides, chymotryptic peptides, lysyl endopeptidase peptides, and CNBr fragments. The Pe-lysozyme was first digested with trypsin. The digested peptides from the soluble fraction (Ts) were separated by RP-HPLC (Fig. 2). The digest yielded 25 peptides as indicated by the number on each peak that proved to contain
Fig. 3. Complete Amino Acid Sequence of Cassowary Lysozyme.

Tryptic peptides (Ts), Lysyl endopeptidase peptides (L), V8 protease peptides (V), chymotryptic peptides (C), and cyanogen bromide fragments (CB).

peptide(s) by amino acid analysis. Some peaks (Ts1, Ts3, Ts5, and Ts11), contained two or three peptides that were further separated by rechromatography in the same column using Solvent system 2. The amino acid compositions of purified tryptic peptides were analyzed. All of these peptides were also sequenced by the Edman sequencing method. The amino acid sequences of tryptic peptides are shown in Fig. 3 as Ts. The sequenced parts of peptides were indicated as solid lines while obtained regions of peptides judged by their amino acid compositions were indicated as dotted lines, with the notable exception of Ts14, which yielded no N-terminal. To analyze the N-terminal sequence, the tryptic peptide (Ts14), which carried a blocked N-terminal amino acid, was analyzed by mass spectrometry. The obtained molecular mass of the N-terminal amino acid corresponded to a pyroglutamate group. The sequence of Ts14 found by LC/MS/MS spectrometry is PyroGlu-Thr-Gly-Cys-Tyr-Gly-Val-Val-Asn-Arg. We confirmed the result from mass spectrometry by further digestion of Ts14 with PGAP. The digested peptide was sequenced and yielded nine amino acid residues as: Thr-Gly-Cys-Tyr-Gly-Val-Val-Asn-Arg. The tryptic peptides were comprised of 183 amino acids. When compared with the other reported G-type lysozymes, this lysozyme should have an extra two amino acids in the polypeptide chain. To obtain undetermined regions and to get the overlapping peptides, Cm-lysozyme was next digested with V8 protease. The peptides from the soluble fraction of the digest were purified and analyzed in the same manner as the tryptic peptides. The V8 protease digest yielded seven overlapping peptides (V16, V25, V31, V38, V39, V42, and V48) (Figure not shown). However nonspecific cleavage by V8 protease were observed after Lys and Arg residues (V25, V31, V42, and V48). The amino acid compositions and amino acid sequences of the V8 protease peptides that overlapped with the tryptic peptides were analyzed. V16 overlapped Ts9 and Ts19, V25 overlapped Ts6 and Ts9, V31 overlapped Ts1b and Ts15, V38 overlapped Ts5a and Ts13, V39 overlapped Ts14 and Ts11b, V42 overlapped Ts1a, Ts3a, and Ts18, V48 overlapped Ts1b, Ts20, and Ts1a, which contained an undetermined Lys residue at position 54, V39 located at the N-terminal of this protein was sequenced after deblocking (Fig. 3). To get other overlapping peptides and undetermined amino acids, chymotrypsin digestion was done on Cm-lysozyme. We obtained nine over-
lapping peptides and their amino acid compositions and amino acid sequences were analyzed. Nine chymotryptic peptides overlapped thirteen tryptic peptides, some overlapping regions were not directly sequenced, but the deduced amino acid sequence judged by the amino acid composition were identical to those of the regions of tryptic peptides (Fig. 3). Lysyl endopeptidase was next used to digest Pe-
lysozyme. Two overlapping peptides were obtained and their amino acid sequences and amino acid compositions were analyzed. L18 peptide overlapped between Ts20 and Ts1a, and L19 overlapped between Ts9 and Ts19 (Fig. 3). To complete the overlapping, Cm-lysozyme was next cleaved with CNBr. This cleavage yielded three overlapping fragments, CB2, CB4, and CB14 judged by their amino acid compositions. These fragments were sequenced and all of the overlapping for tryptic peptides was completed. Namely CB2 overlapped between Ts19 and Ts3b including Lys126, CB4 overlapped between Ts18 and Ts6, and CB14 overlapped Ts16, Ts2, and Ts11a. All of these peptides are aligned in Fig. 3. The amino acid composition of each peptide was proved to be identical with the amino acid sequence found for this enzyme.

N-terminal sequence analysis of cassowary lyso-
zyme from transblotted protein and Cm-cassowary lysozyme yielded no N-terminal amino acid. Hence the N-terminal residue of this protein is assumed to be blocked.33) This study proved that a pyroglutamyl group blocked the N-terminal of the cassowary lyso-
zyme. The N-terminal residue of reported G-type lysozymes from black swan, ostrich, and goose is Arg. No evidence of blocking has been reported in this enzyme group. To date, no lysozyme (C-type, T4-type, or G-type) has been found to contain any blocking groups at the N-terminal. This study is the first evidence for N-terminal blocking in this enzyme group, thus suggesting that cassowary lysozyme has a unique post-translational modification process different from that of the other G-type lysozymes. The goose lysozyme gene from the chicken has been sequenced by Nakano and Graft.32) They indicated that the 22 N-terminal amino acids, rich in hydrophobic residues, might act as a signal peptide, which is cleaved between two glycines to generate the mature form of the protein. In case of cassowary lysozyme, an Arg residue was replaced by a Gln residue that was then assumed to be converted to a pyroglutamyl group by processing of glutamate cyclase in the biosynthesis pathway of pyroglutamate.

Combining the N-terminal sequence analysis, tryptic peptides sequencing, and overlapping pep-
tides sequencing from each enzyme, we established the complete amino acid sequence of cassowary G-
type lysozyme (Fig. 3).

The locations of disulfide bonds

The locations of disulfide bonds were found by a modification of the method of Sueyoshi et al.38) Cassowary lysozyme was digested with acid protease and thermolysin and then separated into S-S peptides by RP-HPLC. S-S bonds were detected using SBD-F, which produces fluorescence when reacting with a SH group. The fluorescence intensity was estimated based on the external standard of GSSG because the fluorescence intensity is proportional to the molar ra-
tio of the SH group.38) The elution profile of the acid protease/thermolysin treated cassowary lysozyme and the fluorescence intensity of each peak is shown in Fig. 4. The resulting six peaks (A-F) that have high fluorescence intensity were rechromatographed to produce single peaks (chromatogram not shown). The purified six S-S peptides were then oxidized by performic acid and further purified into each cysteic acid peptide on the same column. All six peptides were separated into two peptides after oxidation. Amino acid compositions of the purified cysteic acid peptides were analyzed (data not shown). The amino acid compositions of peptide Aa and Ab, and Fa and Fb are in agreement with the Gln1-Cys4 and Leu59-Ala64, and Gln1-Gly6 and Leu59-Ala64 sequences, respectively. This indicates that peptides Aa and Fa contain Cys4 and Ab and Fb contain Cys60. The amino acid compositions of peptide Ba and Bb, Ca and Cb, Da and Db, and Ea and Eb were derived from the Ala16-Thr20 and Leu26-Gly30, Gly15-Thr20 and Leu26-Gly30, Thr14-Thr20 and Leu26-G-
Cassowary Lysozyme

The disulfide-bond-containing peptides as shown in Fig. 4 were oxidized and each resulting single-chain peptide purified by RP-HPLC was sequenced.

Table 1. Sequence Similarity (%) and Number of Amino Acid Substitutions Between Cassowary Lysozyme and Other G-Type Lysozymes

<table>
<thead>
<tr>
<th>Lysozyme</th>
<th>Amino acid substitutions</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken gene</td>
<td>40</td>
<td>78.4</td>
</tr>
<tr>
<td>Black swan</td>
<td>34</td>
<td>81.6</td>
</tr>
<tr>
<td>Goose</td>
<td>31</td>
<td>83.2</td>
</tr>
<tr>
<td>Ostrich</td>
<td>17</td>
<td>90.8</td>
</tr>
</tbody>
</table>

All of the cysteic-acid-containing peptides were completely sequenced including cysteic acid residues (Fig. 5). Peptides Aa and Fa yielded no N-terminal, which indicated that the location of these two peptides is at the N-terminal part of this protein, judged from the position and from carrying the blocked N-terminal as well as native protein. Two disulfide bonds were found in the N-terminal region of the molecule and the location of that S-S bond was indicated in Fig. 6. This is the first report regarding to the location of disulfide bonds in G-type lysozyme found by a chemical method.

Comparison of primary structure

Cassowary lysozyme was composed of 185 amino acid residues with a pyroglutamyl group at N-terminal residue. The molecular mass of cassowary lysozyme calculated from amino acid sequence was 20408 Da, which coincided with the value from SDS-PAGE. The established amino acid sequence of cassowary lysozyme was compared with G-type lysozymes (goose, black swan, ostrich, excluding the G-type lysozyme from the chicken gene) (Fig. 6). Seven substitutions in cassowary lysozyme were newly detected in this enzyme group at PyroGlu1, Glu19, Gly40, Asp82, Thr102, Thr156, and Asn167, indicated by closed triangle in the figure. The substitutions found when cassowary lysozyme is compared to goose lysozyme sequence are boxed. The sequence similarity and the numbers of amino acid substitutions of cassowary lysozyme compared to other reported G-type lysozymes were summarized in Table 1. Cassowary lysozyme had 34, 31, and 17 substitutions from black swan, goose, and ostrich, respectively. Further the goose lysozyme gene from chicken showed 78.4% similarity to cassowary lysozyme. The sequence differences found in these lysozymes may contribute to differences in tertiary structure.

From the information of X-ray crystallography studied by Weaver et al., goose lysozyme bound trisaccharide at subsites B, C, and D. They found that Glu73 corresponded to Glu35 of chicken lysozyme (and to Glu11 of T4 lysozyme), however no Asp residue counterpart for Asp52 in chicken lysozyme was found. Taking this information into account, the catalytic amino acid (Glu73) residue of all reported G-type lysozymes is conserved. The substituted amino acids that might be important in substrate binding were localized in subsite B (Asn122Ser, Phe123Met). These substitutions at subsite B were also found in ostrich. In the case of black swan, only Asn122 was substituted for Asp. For subsite B, an important contribution to substrate binding was ascribed to His101, Asn122, and Phe123, based on hydrogen bond and/or van der Waals interaction with the ring of substrate. Therefore in cassowary lysozyme Ser122 and Met123 might be able to participate in different interactions comparable with those of goose lysozyme. Especially Phe123 may interact with substrate by ring-ring hydrophobic interaction as shown by Trp62 and Trp63 in subsite B of C-type lysozyme. The X-ray structure of goose lysozyme revealed several α-helices: Cys18-Pro23 (H1), Val31-Glu39 (H2), Gln43-Asp46 (H3), Thr50-Leu59 (H4),...
From the comparison of amino acid sequences, we found high conservation of amino acid sequences that formed three of $\alpha$-helices H5, H7, and H8 in all four G-type lysozymes. These three $\alpha$-helices were located at the center of this protein molecule with low B-factor (temperature factor), suggesting the formation of a hydrophobic core in the overall structure of this enzyme group (Fig. 7).\(^6\) In the hydrophobic core, H5 carries catalytic Glu73 at the C-terminal region supposed necessary for the high pK value as that functions as a proton donor in catalytic mechanism. The three strands of $\beta$-sheet B1, B2, and B3 were also highly conserved in all four G-type lysozymes, and may contribute to the formation of an E-G site such as the left side of the E-F site of chicken lysozyme.

Monzingo et al.\(^7\) reported that two helices and a three-stranded $\beta$-sheet, which form the substrate binding and catalytic cleft, are the only invariant el-
ments of the secondary structure among chitinase, chitosanase, and the three lysozymes. From this information we compared the conservation of the three \( \alpha \)-helices of G-type lysozymes (H5, H7, and H8) with that of barley chitinase. Conservation of position 64–73 (H5) resembled the C \( \alpha \)-helix of chitinase (position 48–68). This region might be important for the formation of a catalytic site (including the catalytic amino acid Glu67 of barley chitinase and Glu73 of G-type lysozymes). Conservation on H7 and H8 of G-type lysozymes and barley chitinase may be due to its importance in the formation of a catalytic cleft. This result implied that G-type lysozymes and barley chitinase possess a similar catalytic mechanism.\(^{15}\) However the detailed mechanism of the reaction might depend on the microenvironment of the catalytic site. Further, we found a high prevalence of negatively charged amino acids (Asp and Glu) in cassowary lysozyme when compared with goose lysozyme. This result may be partly responsible for the acidic pH optimum for lytic activity of the cassowary lysozyme, which was previously reported.\(^{33}\) In agreement with this suggestion, from the amino acid sequence of cassowary lysozyme, we found an increased number of negative charge especially Glu residues when compared with goose lysozyme.

The determined positions of S-S bonds of cassowary lysozyme were completely conserved in goose,\(^{11}\) and black swan lysozymes\(^{8}\) as seen from the crystallography data, which was obtained by the heavy atom binding reaction. In addition, the positions of cysteine residues in the amino acid sequence of ostrich lysozyme are also conserved, which implies that ostrich lysozyme would share the same location of disulfide bonds with other G-type lysozymes. This conservation of the location of disulfide bonds probably indicates similar topology of polypeptide chain folding in this lysozyme group. Conservation of the three \( \alpha \)-helices (H5, H7, and H8) which form a hydrophobic core at the center of this protein molecule with the conservation of the two disulfide bonds may have contributed to the stability of the molecule.

In conclusion, all four G-type lysozymes appear to have conserved in secondary structure the core elements (H5, H7, and H8), the catalytic Glu residues, and the location of disulfide bonds responsible for the maintenance of a similar substrate binding mechanism. However the amino acid substitutions that occur at the substrate binding subsites such as Asn122Ser and Phe123Met found in this work may affect the binding site conformation and change the microenvironment resulting in change in substrate binding energy. The effects of amino acid substitutions, which were involved in subsite microenvironment, will be determined by further analysis of oligosaccharide hydrolysis.

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