

The Amino Acid Sequences of Lady Amherst's Pheasant (*Chrysolophus amherstiae*) and Golden Pheasant (*Chrysolophus pictus*) Egg-white Lysozymes

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The amino acids of Lady Amherst's pheasant and golden pheasant egg-white lysozymes have been sequenced. The carboxymethylated lysozymes were digested with trypsin followed by sequencing of the tryptic peptides. Lady Amherst's pheasant lysozyme proved to consist of 129 amino acid residues, and a relative molecular mass of 14 423 Da was calculated. This lysozyme had 6 amino acids substitutions when compared with hen egg-white lysozyme: Phe3 to Tyr, His15 to Leu, Gln41 to His, Asn77 to His, Gln 121 to Asn, and a newly found substitution of Ile124 to Thr. The amino acid sequence of golden pheasant lysozyme was identical to that of Lady Amherst's pheasant lysozyme. The phylogenetic tree constructed by the comparison of amino acid sequences of phasianoid birds lysozymes revealed a minimum genetic distance between these pheasants and the turkey-peafowl group.

Lysozyme is one of the most investigated hydrolases that catalyzes the hydrolysis of β -1,4 linkages of GlcNAc homopolymer or GlcNAc–MurNAc heteropolymer. This enzyme has been classified into three types, hen-type (chicken-type), goose-type, and phage-type, which correlate genetically with each other but have different amino acid sequences. On account of the highly conserved function of hen-type lysozyme, this protein significantly occurs in vertebrates.^{1–8)} However, according to the amino acid sequence data, the rate of mutation fixation of amino acids in lysozyme was higher during evolution than other universally functional proteins such as cytochrome *c*. Reported amino acid sequences of the avian hen-type lysozymes contain a significant number of amino acid substitutions within the same family and, therefore, will be useful for the classification of species of aves.

Previously, we reported the amino acid sequence of Indian peafowl lysozyme⁹⁾ and indicated that this lysozyme may be considered

as a phylogenetic standard to measure the genetic distance between various closely related species using the amino acid sequence data. In this study we report the amino acid sequences of lysozymes isolated from Lady Amherst's pheasant (*Chrysolophus amherstiae*) and golden pheasant (*Chrysolophus pictus*) egg-white. By comparison of these amino acid sequences with those of other phasianoid birds, a phylogenetic tree of phasianoid birds was constructed.

Materials and Methods

Eggs. Freshly laid Lady Amherst's pheasant, golden pheasant, Indian peafowl, Japanese pheasant, and guineafowl eggs were obtained from Kumamoto Zoological Park, Kumamoto, Japan.

Purification of lysozyme. A water extract of egg white was adjusted to pH 4.0 with HCl to precipitate the acidic protein. The pH 4.0 supernatant was adjusted to pH 8.0 with NaOH and the precipitate was removed by centrifugation. The pH 8.0 supernatant was then put on a CM-Toyopearl column equilibrated with 0.03 M phos-

phate buffer, pH 8.0. After this was washed with the same buffer, the stepwise elution with 0.1 M NaCl and 0.3 M NaCl in the same buffer were then done. The lysozyme fraction was eluted with 0.3 M NaCl. The crude lysozyme fraction was then rechromatographed on the same column using a gradient of 0.1 M to 0.3 M NaCl in the phosphate buffer. The details of the preparation of these lysozymes will be published elsewhere. Hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Japan.

Carboxymethylation. Lysozyme was reduced and carboxymethylated by the method of Crestfield *et al.*¹⁰ Lyophilized lysozyme (5 mg) was dissolved in 300 μ l of 1.44 M Tris-HCl buffer, pH 8.6, followed by addition of 30 μ l of 5% EDTA solution, 0.36 g of urea, and 15 μ l of β -mercaptoethanol, and the mixture adjusted to 750 μ l with water. After a reduction of 1 hr under N₂ at 37°C, the carboxymethylated lysozyme (Cm-lysozyme) was desalted on a Sephadex G-50 column (1.0 $\times$ 30 cm) in 0.2 N NH₄OH and then lyophilized.

Enzymatic digestion. Cm-Lysozyme (5 mg) was dissolved in 1.5 ml of distilled water, adjusted to pH 8.0 with 0.002 N NH₄OH, and digested with 1/50 (w/w) of trypsin (Cooper Biochemical, TR-TPCK) at 37°C, pH 8.0 for 4 hr using a pH stat (Radiometer Co.). The digest was then used directly.

Peptide separation. The tryptic digests were put on a RP-HPLC column (0.4 $\times$ 25 cm, Yamamura Chemical, C18 120A S-5 A) using a JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The peptides were eluted with a gradient system of 0.1% TFA (solvent A) and 60% acetonitrile in 0.1% TFA (solvent B). That is, after injection of 50 μ l of tryptic digest, the column was eluted with solv. A for 10 min and then a linear gradient of 0 to 50% of solv. B was run through for 130 min. Other conditions were as follows: flow rate; 1.0 ml/min, detection wavelength; 220 nm, monitored range; 0.64 AUFS. Each peak was collected manually for further investigation.

Amino acid composition and amino acid sequence analysis. Tryptic peptide was hydrolyzed with constant boiling HCl containing 0.05% β -mercaptoethanol in an evacuated sealed tube at 110°C for 20 hr. The hydrolysate was analyzed with a Hitachi Amino Acid Analyzer, Model 835. The amino acids of peptides were sequenced by the DABITC/PITC double coupling method.^{11,12)}

Results and Discussion

Amino acid sequence of Lady Amherst's pheasant lysozyme

The elution pattern of tryptic peptides from Lady Amherst's pheasant lysozyme (LAPL) was compared with those of hen, Indian

peafowl, Japanese pheasant, and guineafowl lysozymes. Of these patterns, the peafowl lysozyme gave the most similar pattern to LAPL (Fig. 1). The elution positions of peptides except peptides T11, T13, and T16 coincided with those derived from Indian peafowl lysozyme. The elution positions of peptides T11 and T13 coincided with those from Japanese pheasant lysozyme. However, the elution position of peptide T16 did not coincide with that from any lysozymes. The amino acid composition of each tryptic peptide from LAPL is listed in Table I. All of the peptides were then sequenced by the DABITC/PITC method (Fig. 2). The amino acid sequences of all peptides except T11, T13, and T16 identical to those from Indian peafowl lysozyme. Also the amino acid sequences of peptides T11 and T13 were identical to the corresponding peptides from Japanese pheasant lysozyme. The peptide T16 having an amino acid composition unknown in other birds' lysozymes proved to contain a new substitution of Ile124 to Thr. The peptide alignment was deduced by the comparison of elution patterns of LAPL and those from other phasianoid birds.

The amino acid sequence of LAPL had 129 amino acid residues and a molecular mass of 14423, and had 6 amino acid substitutions compared with hen egg-white lysozyme (Fig. 2). Five of 6 amino acid substitutions, Phe/Tyr at position 3, His/Leu at position 15, Gln/His at position 41, Asn/His at position 77, and Gln/His at position 121, have been observed in other birds' lysozymes. However, the substitution of Ile to Thr at position 124 was new. No amino acid exchange in this position occurred in any other birds' lysozyme, and only the exchange of Ile to Val was found in vertebrate lysozymes (human, cow, and baboon).

Amino acid sequence of golden pheasant lysozyme

The elution pattern of tryptic peptides from golden pheasant lysozyme (GPL) was compared with that of LAPL (Fig. 3). The elution

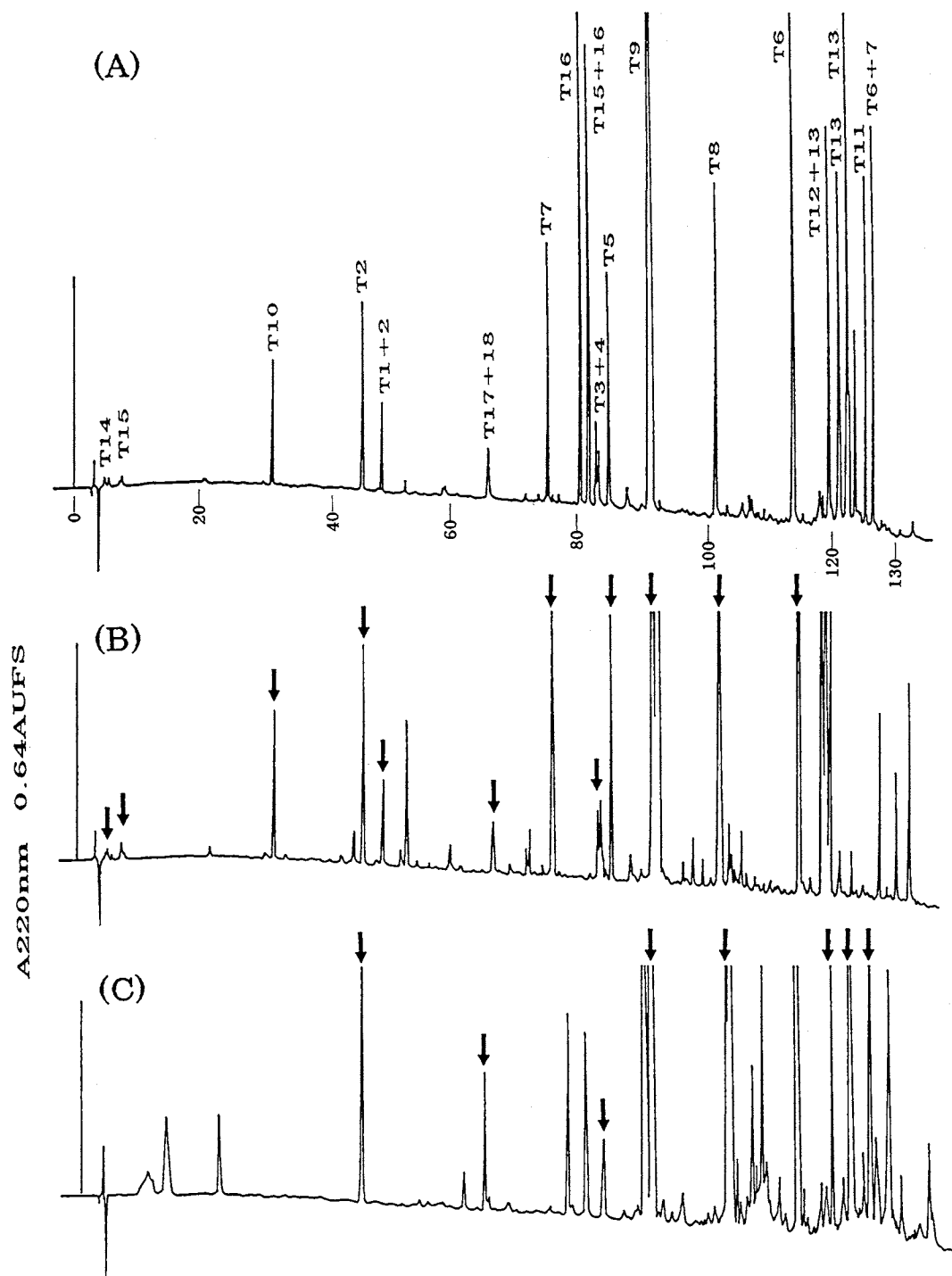


Fig. 1. Comparison of Elution Pattern of the Tryptic Peptides from Lady Amherst's Pheasant Lysozyme with Those from Two Phasianoid Birds.

(A) Lady Amherst's pheasant lysozyme; (B) Indian peafowl lysozyme; (C) Japanese pheasant lysozyme. Arrows on the peaks of Indian peafowl lysozyme and Japanese pheasant lysozyme indicate the peptides eluted on the same position as Lady Amherst's pheasant lysozyme. For conditions of HPLC see Materials and Methods.

Table I. AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES FROM LADY AMHERST'S PHEASANT LYSOZYME

The amino acid compositions different from those of Indian peafowl was indicated by an underline.

	T1+2	T3+4	T5	T6	T7	T8	T9+10	T11	T12+13	T14	T15	T16	T17+18
Asp			2.05 (2)	0.74 (1)	3.03 (3)	3.52 (4)	2.04 (2)	3.05 (3)	2.82 (3)	0.94 (1)		1.89 (2)	
Thr					1.98 (2)	1.90 (2)	1.01 (1)	1.05 (1)				1.92 (2)	
Ser				0.92 (1)	1.00 (1)	1.82 (2)	1.10 (1)	4.17 (4)	1.05 (1)				
Glu		1.04 (1)			1.07 (1)	1.04 (1)							
Pro							1.10 (1)	1.22 (1)					
Gly	1.07 (1)		1.04 (1)	1.90 (2)		1.96 (2)	2.22 (2)		2.05 (2)			1.01 (1)	1.05 (1)
Ala		3.01 (3)		1.91 (2)	1.15 (1)			3.40 (3)	2.09 (2)			1.07 (1)	
Cys ^a		0.97 (1)		0.81 (1)			0.92 (1)	2.93 (3)			0.83 (1)		0.89 (1)
Val	0.98 (1)			1.00 (1)				1.11 (1)	1.63 (2)			0.99 (1)	
Met		0.92 (1)							0.93 (1)				
Ile						1.90 (2)		2.02 (2)	0.81 (1)				
Leu		1.13 (1)	2.20 (2)	1.03 (1)		0.89 (1)		3.43 (3)					1.10 (1)
Tyr	0.93 (1)		1.00 (1)	0.92 (1)		0.98 (1)							
Phe					2.11 (2)								
Lys	0.99 (1)	1.01 (1)		1.00 (1)				1.22 (1)	1.10 (1)		1.00 (1)		
His					0.99 (1)			1.00 (1)					
Arg	1.00 (1)	1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)	2.00 (2)		1.00 (1)	1.00 (1)		1.00 (1)	1.00 (1)
Trp ^b				0.67 (1)			2.10 (2)		1.83 (2)			1.08 (1)	
Total	5	9	7	12	12	16	12	23	16	2	2	9	4

^a Cys was measured as CmCys.^b Trp was calculated by the method of Araki *et al.*⁹⁾

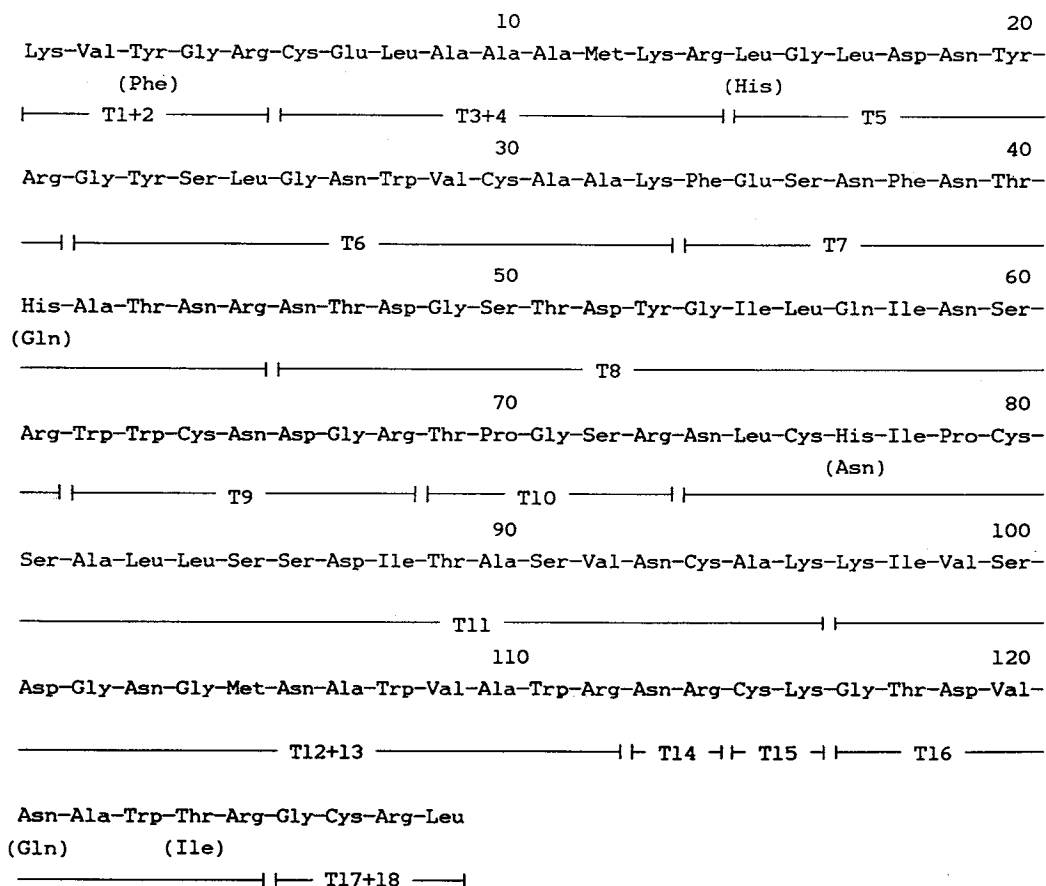


Fig. 2. Amino Acid Sequence of Lady Amherst's Pheasant Lysozyme.

The tryptic peptides isolated by HPLC separation as shown in Fig. 1 were sequenced using the DABITC/PITC Edman degradation method. substituted amino acids compared with hen egg-white lysozyme were indicated in the parenthesis under the corresponding residues.

position of each peptide of GPL coincided with that of the corresponding peptides of LAPL. It could be concluded that the amino acid sequence of the tryptic peptides from GPL are identical to those of the corresponding peptide from LAPL. Some peptides (T8, T6 + 7), which had a slight difference in their elution positions between two lysozymes at the end of the HPLC run, eluted in the same position by another HPLC using a mixture of tryptic digests of these two lysozymes in the same ratio under the same condition (chromatograms not shown). By amino acid analysis, the tryptic peptides of GPL were identical to those from LAPL (Table II). These results strongly support the conclusion that these two lyso-

zymes have identical amino acid sequences.

Comparison of amino acid sequences of Lady Amherst's pheasant and golden pheasant lysozymes with those from other phasianoid birds

The amino acid sequences of LAPL and GPL were compared with those of 9 lysozymes from other phasianoid birds (Fig. 4). Threonine at position 124 was only found in LAPL and GPL, and not in other lysozymes. The amino acid sequences of LAPL and GPL were homologous to that of peafowl lysozyme. In these 9 lysozymes, 23 exchangeable positions were found. Among these positions, the amino acids at positions 15, 41, 102, and 121 contained

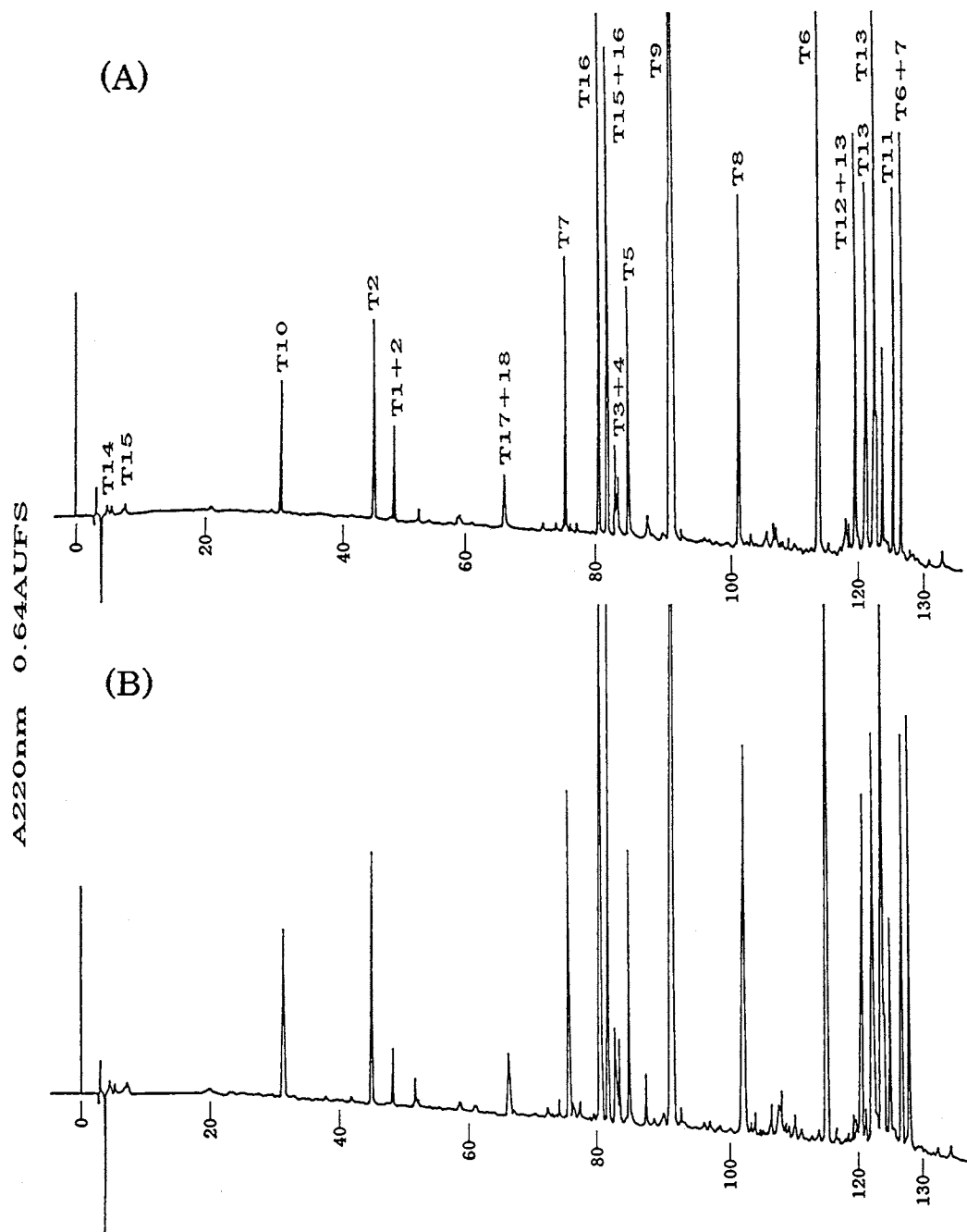


Fig. 3. Comparison of Elution Pattern of Lady Amherst's Pheasant Lysozyme (A) and Golden Pheasant Lysozyme (B).

Conditions of HPLC were the same as in Fig. 1.

substitutions caused by 2 point mutations and are considered to be hypervariable positions.⁹⁾ The lack of amino acid substitution between

two lysozymes from Lady Amherst's pheasant (*Chrysolophus amherstiae*) and golden pheasant (*Chrysolophus pictus*) suggests that the lyso-

Table II. AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES FROM GOLDEN PHEASANT LYSOZYME

	T1+2	T3+4	T5	T6	T7	T8	T9+10	T11	T12+13	T14	T15	T16	T17+18
Asp			1.87 (2)	0.78 (1)	2.99 (3)	3.92 (4)	2.07 (2)	3.05 (3)	3.28 (3)	1.07 (1)		1.88 (2)	
Thr					1.96 (2)	1.93 (2)	0.98 (1)	0.98 (1)				1.93 (2)	
Ser				0.95 (1)	0.97 (1)	1.87 (2)	1.05 (1)	3.68 (4)	1.25 (1)				
Glu		1.01 (1)			1.04 (1)	1.04 (1)							
Pro							1.04 (1)	1.00 (1)					
Gly	1.07 (1)		1.06 (1)	1.89 (2)		2.05 (2)	2.31 (2)		2.30 (2)			1.05 (1)	1.13 (1)
Ala		2.81 (3)		1.87 (2)	1.06 (1)			3.06 (3)	2.27 (2)			1.11 (1)	
Cys ^a		0.94 (1)		0.77 (1)			0.92 (1)	2.62 (3)			0.71 (1)		
Val	1.22 (1)			0.87 (1)				1.06 (1)	1.78 (2)				0.98 (1)
Met		0.89 (1)							0.86 (1)			1.03 (1)	
Ile						1.99 (2)		1.87 (2)	0.73 (1)				
Leu		1.09 (1)	2.19 (2)	1.00 (1)		0.89 (1)		3.16 (3)					1.13 (1)
Tyr	1.01 (1)		1.00 (1)	0.93 (1)		1.03 (1)							
Phe					2.06 (2)								
Lys	0.92 (1)	1.00 (1)		1.00 (1)				1.00 (1)	1.00 (1)		1.00 (1)		
His					0.99 (1)			0.94 (1)					
Arg	1.00 (1)	0.56 (1)	1.00 (1)		1.00 (1)	1.00 (1)	2.00 (2)		0.98 (1)	1.00 (1)		1.00 (1)	1.00 (1)
Trp ^b				0.70 (1)			2.10 (2)		1.97 (2)			1.00 (1)	
Total	5	9	7	12	12	16	12	23	16	2	2	9	4

^a Cys was measured as CmCys.

^b Trp was calculated by the method of Araki *et al.*⁹⁾

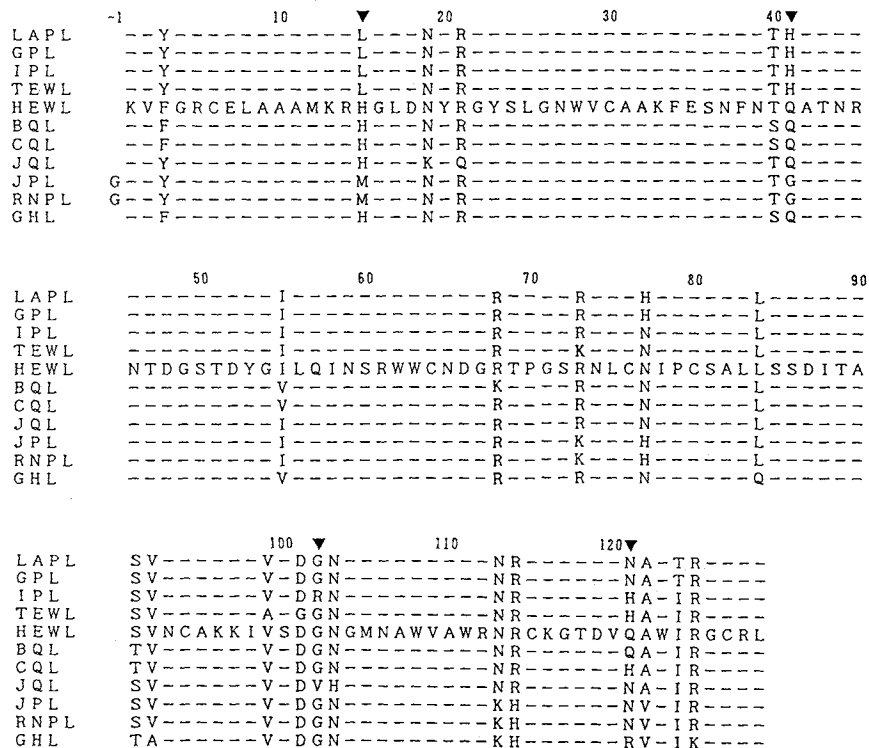


Fig. 4. Amino Acid Sequence Comparison of Lysozymes from Phasianoid Birds.

Abbreviations are as follows: LAPL, Lady Amherst's pheasant lysozyme; GPL, golden pheasant lysozyme; IPL, Indian peafowl lysozyme⁹; TEWL, turkey egg-white lysozyme^{13,14}; HEWL, hen egg-white (chicken) lysozyme^{1,2}; BQL, bobwhite quail lysozyme¹⁵; CQL, California quail lysozyme¹⁶; JQL, Japanese quail lysozyme¹⁷; JPL, Japanese pheasant lysozyme¹⁸; RNPL, ring-necked pheasant lysozyme¹⁹; GHL, guinea fowl lysozyme²⁰. Amino acid sequence were represented by single letters on HEWL and the positions carrying the exchanged amino acids were indicated for all lysozymes. Nonsubstituted positions were indicated by dashes. Arrows indicate the hypervariable positions containing substituted amino acids with 2 point mutations.

zymes from the same species have the same amino acid sequence. The same amino acid sequence has also been found in the species of *Phasianus* (Japanese pheasant and ring-necked pheasant).¹⁸ In the sequence comparison of LAPL and GPL with hen egg-white lysozyme, no amino acid substitution occurred near the active cleft of LAPL and GPL.

The homology percent of the amino acid sequence and combined minimum base change value of substituted amino acids between two lysozymes are summarized in Fig. 5. A phylogenetic tree of phasianoid birds was constructed by the unweighted pair-group method using the minimum base change value (Fig. 6). Lady Amherst's pheasant was shown to have a minimum genetic distance to the

turkey-peafowl group, followed by the chicken-quail group. The relative position of each bird in this phylogenetic tree did not coincide with the general classification of phasianoid birds.²¹ Turkey and guinea fowl have been classified as meleagridinae and numidinae, respectively, and two quails, California and Bobwhite quail, have been classified as odontophorinae, but all other birds have been in phasianinae (shown in the parenthesis in Fig. 6). In contrast, the classification deduced from this phylogenetic tree separates these birds into two groups; Japanese pheasant, ring-necked pheasant, and guinea fowl group, and the other 7 birds group, by the genetic distance calculated from the minimum base change value. The significance of the difference between the morphological

	LAPL	GPL	IPL	TEWL	HEWL	JPL	RNPL	JQL	CQL	BQL	GHL	
	0	0	4	6	6	8	8	8	9	10	16	LAPL
		0	4	6	6	8	8	8	9	10	16	GPL
LAPL	100		0	4	5	10	10	8	7	9	14	IPL
GPL	100	100		0	7	10	10	10	9	11	16	TEWL
IPL	97	97	100		0	13	13	7	4	4	10	HEWL
TEWL	95	95	97	100		0	0	14	15	17	15	JPL
HEWL	95	95	96	95	100		0	14	15	17	15	RNPL
JPL	95	95	93	93	93	100		0	8	10	15	JQL
RNPL	95	95	93	93	93	100	100		0	2	7	CQL
JQL	94	94	95	92	95	91	91	100		0	8	BQL
CQL	93	93	95	93	97	91	91	94	100		0	GHL
BQL	92	92	93	91	97	90	90	93	98	100		
GHL	88	88	89	88	92	91	91	88	95	94	100	
	LAPL	GPL	IPL	TEWL	HEWL	JPL	RNPL	JQL	CQL	BQL	GHL	

Fig. 5. Sequence Homology and Minimum Base Change Matrices of Phasianoid Birds' Lysozymes.

Lower left indicates the sequence homology percent and upper right indicates the minimum base change values. For abbreviations see Fig. 4. An extra Gly residue at N-terminus of Japanese pheasant and ring-necked pheasant lysozymes which was considered to be a product by the splicing occurred at the different position from other lysozymes,¹⁹⁾ was not counted in this study.

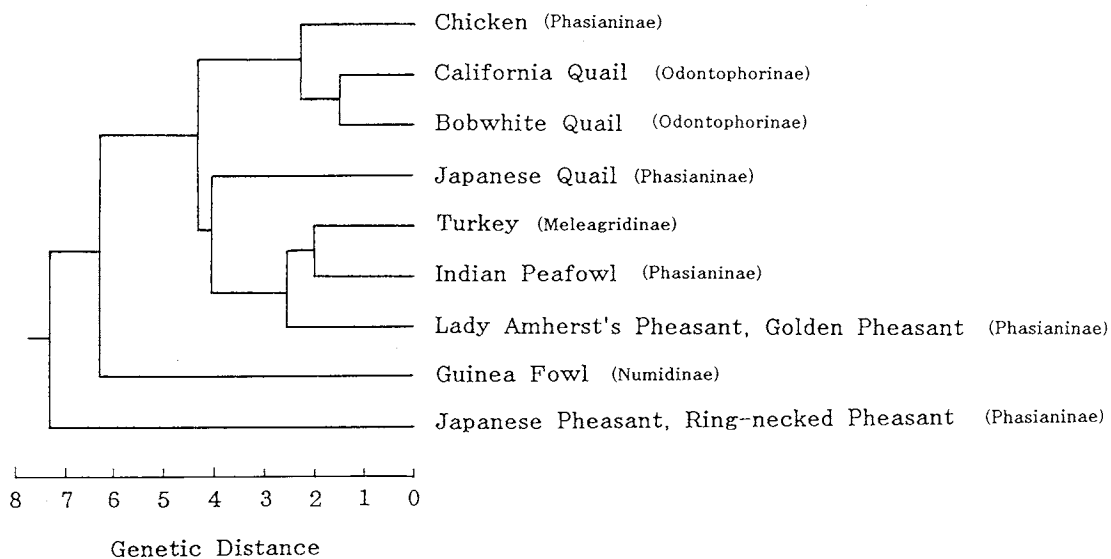


Fig. 6. Phylogenetic Tree of Phasianoid Birds Constructed with Minimum Base Change Values of Amino Acid Sequence of Lysozyme.

Minimum base change value of each group was indicated as genetic distance in the figure. Subfamily name to which each bird belongs is indicated in the parenthesis.

classification and a molecular biological classification requires further investigation. However, the information derived from the proteins that have high evolutionary rates such as lysozyme is expected to be a phylogenic standard as a representative form of the genetic

evolutionary process of organisms to classify closely related species.

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