

The Amino Acid Sequence of Lysozyme from Kalij Pheasant (*Lophura leucomelana*) Egg-white

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The amino acid sequence of kalij pheasant lysozyme has been analyzed. From the comparison of the tryptic peptide pattern of kalij pheasant lysozyme and maps from other bird lysozymes followed by the sequencing of tryptic peptides, the amino acid sequence of kalij pheasant was found to be: KVGRCELAAAMKRLGLDNYRGYSLGNWVCAAKYESNFNTHATNRNTDGGSTDYGILQINSRWWCNDGKTPGSRNLCHIPCSALLSSDITASVNC AKKIVSDGNGMNAWVAWRNRCKGTDVSVWTRGCRL. This sequence had 9 amino acid substitutions compared with hen egg-white lysozyme. Two of these substitutions, positions 34 and 121, were newly detected in phasianid birds. The protein genealogy of phasianid bird lysozymes showed some discordance with the morphological classification of these birds.

In some proteins, nonuniformity in the evolutionary rate has been observed. However, the evolutionary rate is independent from other proteins of individuals.^{1,2)} Therefore, the comparison of the amino acid sequence data of certain proteins is useful for their phylogenetic classification. For this concept, cytochrome *c* has been used for the strategy to elucidate the genetical relationship of organisms.³⁾ The required conditions of this method are; (1) the protein must exist in all kinds of organisms and (2) the amino acid sequence of this protein must be easy to analyze. However, cytochrome *c* study did not classify closely related animals due to a low evolutionary rate. Use of a protein with a high evolutionary rate is expected to solve this problem in terms of molecular genetics.

Recently, we pointed out that lysozyme would be available as a phylogenetic standard of classification of closely related birds in a family with the rapid analysis of the amino acid sequence by comparison of peptide maps of

tryptic peptides.^{4,5)} In spite of the highly conserved function of this enzyme, the rate of mutation fixation of this protein is 7 times higher than that of cytochrome *c*,²⁾ namely, reported amino acid sequence of avian hen-type (C-type) lysozyme contain 3 to 10 amino acid substitutions within the same family. Therefore lysozymes from closely related birds contain sufficient information on species to evaluate their lineage.

In this study, we report the amino acid sequence of kalij pheasant (*Lophura leucomelana*) lysozyme (KPL) and compare it with those of other phasianid bird lysozymes and discuss the phylogenetic relationship between phasianid birds.

Materials and Methods

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

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Abbreviations: DABITC, 4-*N,N*-dimethylaminoazobenzene 4'-isothiocyanate; PITC, phenylisothiocyanate; TFA, trifluoroacetic acid; RP-HPLC, reversed phase high performance liquid chromatography; KPL, kalij pheasant.

Purification of lysozyme. A water extract of egg white was adjusted to pH 4.0 with HCl to remove acidic proteins. The resulting supernatant was adjusted to pH 8.0 with NaOH, put on a CM-Toyopearl column equilibrated with 0.03 M phosphate buffer, pH 8.0, and eluted with the same buffer containing 0.3 M NaCl. The lysozyme fraction was then rechromatographed using a same column but with a gradient of 0.1 M to 0.3 M NaCl in the same buffer. Hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Japan.

Peptide separation. Carboxymethylated⁶⁾ KPL was digested with trypsin and the resulting peptides were separated by RP-HPLC.⁵⁾ Briefly, carboxymethylated lysozyme was digested with 1/50 (w/w) trypsin (TR-TPCK, Cooper Biomedical Co.) at 37°C, pH 8.0 for 4 hr. The tryptic digest was put onto a HPLC (JASCO 800 Series HPLC, Japan Spectroscopic Co., Japan) with a reverse-phase (RP) HPLC column (C18, 120 Å S-5, 4.0 × 250 mm, Yamamura Chemical Co., Japan). The tryptic peptides were developed with a gradient elution system of 0.1% TFA (solv. A) and 60% acetonitrile in 0.1% TFA (solv. B). The gradient of 0% to 50% of solv. B was used for 130 min after 10 min elution with solv. A. The resulting elution pattern was compared with those of other bird lysozymes prepared by the same procedure and the sequences of the collected peptides were analyzed.

Amino acid analysis and amino acid sequencing. Tryptic peptides were hydrolyzed in an evacuated sealed tube at 110°C for 20 hr with constant-boiling HCl containing 0.05% β-mercaptoethanol. Resulting hydrolysates were analyzed using an amino acid analyzer (Model 835, Hitachi Co., Japan). The amino acid sequence of the tryptic peptides were analyzed using the DABITIC/PITC double coupling micro sequencing method.^{7,8)}

Construction of phylogenetic tree. The phylogenetic trees were constructed by the unweighted pair-group clustering method⁹⁾ using three different distance values: the minimum base change (MBC), the mutation data matrix (MDM) reported by Schwartz *et al.*¹⁰⁾ and the distance matrix (DM) reported by Risler *et al.*¹¹⁾

Results and Discussion

The elution pattern of KPL on RP-HPLC was compared with those of hen, Indian peafowl, Lady Amherst's pheasant, Japanese pheasant, and guineafowl lysozyme, respectively. Homologous HPLC patterns were obtained from KPL and from Lady Amherst's pheasant lysozyme (LAPL) as shown in Fig. 1. The elution position of each peak was compared between two patterns. The following

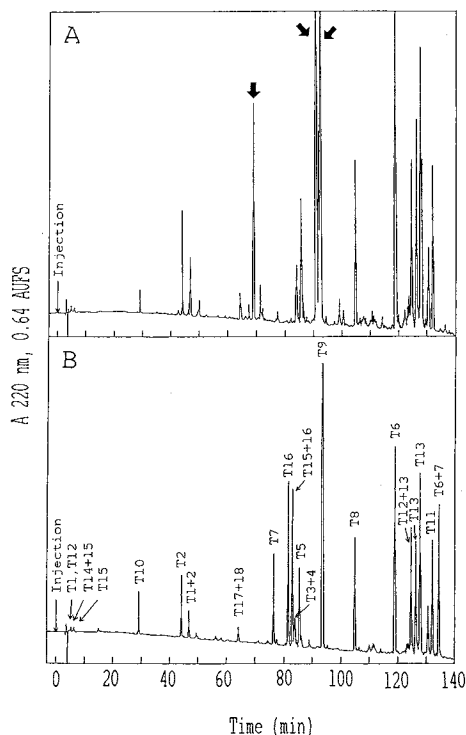


Fig. 1. Comparison of Elution Patterns of Kalij and Lady Amherst's Pheasants.

The elution pattern of KPL (A) obtained by RP-HPLC was compared with other patterns of phasianid bird lysozymes. The HPLC pattern of LAPL (B) is represented in this figure for comparison. The peptides showing different elution positions between two patterns are indicated by arrows in the figure. For the conditions of HPLC see text.

points should be noted. All peptides except peptides T7, T16, T15+16, T9, T9+10, and T6+7 were demonstrated to have identical elution positions between KPL and LAPL, and therefore they were assumed to have identical amino acid sequences. Two peaks of peptide T13 found in KPL were generally found in order lysozymes and it has been demonstrated to be due to deamidation of Asn 103. The peptides eluted at different positions between the two maps indicated by arrows in the figure were looked for in other bird lysozymes. However no identical peptide was found and it indicated that these peptides have different amino acid sequences from the corresponding peptides of the lysozymes we compared.

Table I. COMPARISON OF AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES FROM KALIJ PHEASANT LYSOZYME AND LADY AMHERST'S PHEASANT LYSOZYME
The differences of amino acid compositions between two lysozymes are underlined.

	T7		T9		T9+10		T15+16		T16	
	KPL	LAPL	KPL	LAPL	KPL	LAPL	KPL	LAPL	KPL	LAPL
Asp	3.06(3)	3	1.97(2)	2	2.04(2)	2	1.22(1)	2	1.02(1)	2
Thr	2.01(2)	2			1.03(1)	1	2.01(2)	2	1.83(2)	2
Ser	1.04(1)	1			1.06(1)	1	1.11(1)	0	0.87(1)	0
Glu	1.10(1)	1								
Pro										
Gly			0.94(1)	1	2.19(2)	2	1.10(1)	1	1.10(1)	1
Ala	1.12(1)	1						1		1
CmCys			0.85(1)	1	0.96(1)	1	1.00(1)	1		
Val							2.23(2)	1	2.02(2)	1
Met										
Ile										
Leu										
Tyr	1.07(1)	0								
Phe	1.04(1)	2								
Lys			1.00(1)	0	1.14(1)	2	1.24(1)	1		
His	1.04(1)	1								
Arg	1.00(1)	1		1	1.00(1)	0	1.00(1)	1	1.00(1)	1
Trp			1.71(2)	2	1.79(2)	2	0.92(1)	1	0.88(1)	1
Total	12	12	7	7	11	11	11	11	9	9

The completion of an amino acid sequence was done as follows: the amino acid compositions or tryptic peptides were compared with those from LAPL. Only peptides T7, T16, T15+16, T9, T9+10, and T6+7 were found to have different amino acid compositions than that of LAPL as shown in Table I, while the other peptides have been shown to have identical amino acid compositions in these two lysozymes. Subsequently all peptides were sequenced to analyze the whole sequence.

The established amino acid sequence of KPL was compared with 11 reported amino acid sequences of phasianid bird lysozymes (Fig. 2). The following feature of the amino acid sequence of KPL were noted. Two characteristic amino acid substitutions, Tyr34 and Ser121, were found in KPL. Tyrosine in position 34 was not found in other phasianid bird lysozymes, but it is reported to exist in duck lysozymes.^{21,22} Also serine in position 121 were not found in other phasianid bird lysozymes, but these amino acid substitutions were found in duck lysozymes and in vertebrate

lysozymes (baboon, cow, goat, deer, and rat). The amino acid residues forming the active site of lysozyme of KPL were not changed from that of hen egg-white lysozyme.

The protein genealogies of twelve phasianid bird lysozymes (10 species) were constructed by the three different distance parameters. The genealogies constructed by the values of MBC, MDM, and DM are shown in Fig. 3A, 3B, and 3C, respectively. It should be noted a variety of 103th amino acid residue, Asn103, or Asp103, in lysozyme sequences was considered to be due to post biosynthesized deamidation, the reported Asp103 were changed to Asn for calculation of genetic distance in this study.

The constructed genealogical trees showed that these lysozymes were apparently classified into two groups: the chicken-quail group and the pheasant group, as shown in Fig. 3. However the detailed lineage of lysozymes from the chicken-quail group is different from each other in these protein genealogies. Especially the tree constructed by DM value classified guineafowl into the chicken-quail group,

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K P L		K	V	Y	G	R	C	E	L	A	A	A	M	K	R	L	G	L	D	N	Y	R	G	Y	S	L	G	N	W	V	C	A	A	K	Y	E
L A P L, G P L		--	Y	--	--	--	--	--	--	--	--	--	--	--	--	L	--	--	N	--	R	--	--	--	--	--	--	--	--	--	--	--	--	--	F	--
I P L		--	Y	--	--	--	--	--	--	--	--	--	--	--	--	L	--	--	N	--	R	--	--	--	--	--	--	--	--	--	--	--	--	--	F	--
T E W L		--	Y	--	--	--	--	--	--	--	--	--	--	--	--	L	--	--	N	--	R	--	--	--	--	--	--	--	--	--	--	--	--	--	F	--
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K P L		S	N	F	N	T	H	A	T	N	R	N	T	D	G	S	T	D	Y	G	I	L	Q	I	N	S	R	W	W	C	N	D	G	K	T	P
L A P L, G P L		--	--	--	T	H	--	--	--	--	--	--	--	--	--	--	--	--	--	I	--	--	--	--	--	--	--	--	--	--	--	--	--	--	R	--
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J Q L		--	--	--	T	Q	--	--	--	--	--	--	--	--	--	--	--	--	--	I	--	--	--	--	--	--	--	--	--	--	--	--	--	--	R	--
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L A P L, G P L		--	R	--	--	H	--	--	--	--	--	--	--	--	L	--	--	--	--	--	S	V	--	--	--	--	--	--	--	V	--	D	G	N	--	--
I P L		--	R	--	--	N	--	--	--	--	--	--	--	--	L	--	--	--	--	--	S	V	--	--	--	--	--	--	--	V	--	D	R	N	--	--
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H E W L		--	R	--	--	N	--	--	--	--	--	--	--	--	L	--	--	--	--	--	S	V	--	--	--	--	--	--	--	V	--	D	G	N	--	--
J P L, R N P L		--	K	--	--	H	--	--	--	--	--	--	--	--	L	--	--	--	--	--	S	V	--	--	--	--	--	--	--	V	--	D	G	N	--	--
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L A P L, G P L		--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	N	A	--	T	R	--	--												
I P L		--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	N	A	--	I	R	--	--												
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Fig. 2. Amino Acid Sequence of Kalij Pheasant Lysozyme and Its Comparison with Other Phasianid Bird Lysozymes.

Abbreviations are, KPL; Kalij pheasant lysozyme, LAPL; Lady Amherst's pheasant lysozyme,¹²⁾ GPL; golden pheasant lysozyme,¹²⁾ IPL; Indian peafowl lysozyme,⁵⁾ TEWL; turkey lysozyme,¹³⁾ HEWL; hen lysozyme,^{14,15)} JPL; Japanese pheasant lysozyme,⁴⁾ RNPL; ring-necked pheasant lysozyme,¹⁶⁾ BQL; bobwhite quail lysozyme,¹⁷⁾ CQL; California quail lysozyme,¹⁸⁾ JQL; Japanese quail lysozyme,¹⁹⁾ GHL; guineafowl lysozyme.²⁰⁾ Positions which contain no amino acid substitutions are indicated by dashes.

although they have a distant genetic relation in the genealogies of MBC and MDM values. The reason of this discordance seems to be that the MDM and DM value is basically evaluated from the frequency of observed amino acid substitutions in related proteins, which is conceptionally different from the MBC value. Further, the DM value does not contain the conception of any chemical nature of the amino acid side-chain but the structure of homologous

protein (counting an exchangeable pair those residues whose C α atoms are very close to one another after superposition of the structure) which indicated that this distance matrix is better than any other published one for aligning the sequences of distantly related proteins.¹¹⁾ Therefore, the most probable relationship of these bird lysozymes may be represented by the protein genealogy constructed from the DM value.

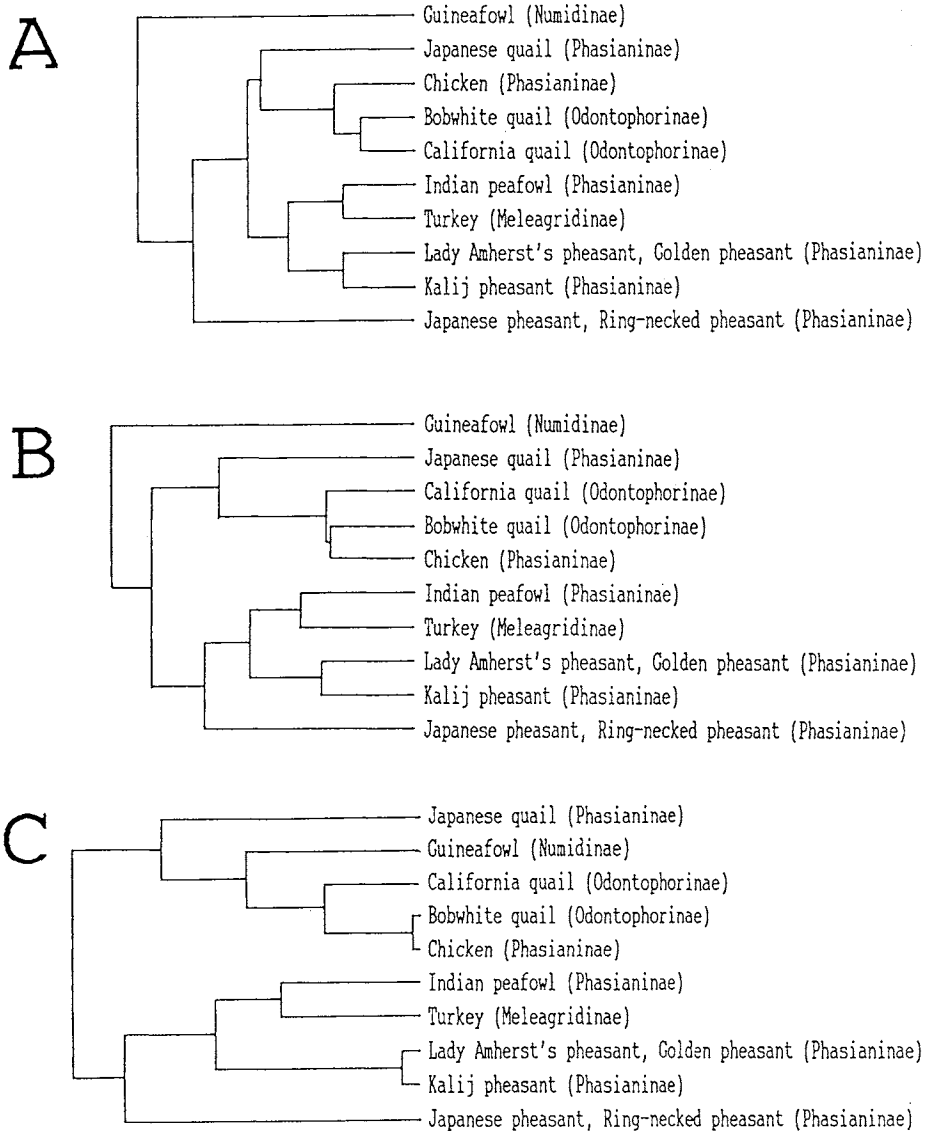


Fig. 3. Protein Genealogy of Phasianid Bird Lysozymes Constructed by Minimum Base Change Value (A), Mutation Data Matrix Value (B), and Distance Matrix Value (C) of Amino Acid Sequences.

The subfamily of each bird from morphological classification of phasianid birds is shown in parentheses.

On the other hand, the classification of phasianid birds by the protein genealogy was not consistent with the classical morphological classification of these birds.²³⁾ Namely, two quails, bobwhite quail and California quail, appeared not to belong to the phasianinae subfamily by the morphological classification. However, they were closely related to chicken (phasianinae) judging by the genetic distance

found in this study. Further, turkey, classified in the meleagridinae subfamily, were found to be closely related to peafowl (phasianinae). The same difference of the classification was also reported in our previous paper.⁵⁾

The difference between the classification of birds of this study and the classical morphological method implies that the protein molecule, which exists as the represented form of the gene

reflecting the evolution of organisms, and the physiological feature which reflects the environment adaptation, are distinct from each other. Combining these results, the taxonomical information obtained by comparison of amino acid sequence of lysozymes is considered to provide new information for the classification of closely related species in the same family.

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