

Note

The Amino Acid Sequence of Copper Pheasant Lysozyme

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The amino acid sequence of copper pheasant lysozyme was analyzed. Carboxymethylated lysozyme was digested with trypsin and the resulting peptides were sequenced. The established amino acid sequence had three amino acid substitutions at positions 20, 77, and 113 for Lady Amherst's pheasant lysozyme and seven amino acid substitutions at positions 3, 15, 20, 41, 113, 121, and 124 for hen lysozyme. Phenylalanine at position 20 was newly detected in avian lysozyme.

C-type (hen-type) lysozyme is the most characterized hydrolase; it selectively cleaves the β 1-4 glycosidic bond of *N*-acetylmuramic acid and *N*-acetylglucosamine. One of the most intensive investigations for amino acid sequences of lysozyme is for avian egg white, especially for Galliformes. The complete amino acid sequences of phasianid birds are known for 12 lysozymes: hen (HEWL),¹⁻³ turkey (TEWL),⁴ bobwhite quail (BQL),⁵ California quail (CQL),⁶ Japanese quail (JQL),⁷ ring-necked pheasant (RNPL),⁸ Japanese pheasant (JPL),⁹ Lady Amherst's pheasant (LAPL),¹⁰ golden pheasant (GPL),¹⁰ kalij pheasant (KPL),¹¹ reeves' pheasant (RPL),¹² Indian peafowl (IPL),¹³ and guineafowl (GHL).¹⁴ As lysozyme has a high evolutionary rate, the comparison of amino acid sequences from closely related birds provides new information. In our previous paper, we reported the hyper-variable regions found in the lysozyme molecule.¹² In this study we report the amino acid sequence of copper pheasant lysozyme (CPL).

Freshly laid copper pheasant eggs were kindly given to us by Dr. Masaki Kobayashi, Saitama Prefectural Poultry Experiment Station, Saitama, Japan. Other eggs were obtained from The Kumamoto Zoological and Botanical Gardens, Kumamoto, Japan. Hen egg-white lysozyme was purchased from Seikagaku Kogyo Co., Japan. A water extract of egg white was treated with isoelectric precipitations at pHs 4.0 and 7.0 and the clarified solution was then put on a CM-Toyopearl column (1.5 × 46 cm) equilibrated with 0.03 M phosphate buffer (pH 7.0). The column was then eluted stepwise with the same buffer containing 0.3 M NaCl. The lysozyme fraction was rechromatographed on the same column with a gradient of 0.1 M to 0.3 M NaCl in the same buffer. Enzyme activity was monitored by a lytic activity using the lyophilized cell wall of *Micrococcus lysodeikticus* as a substrate. Lysozyme solution (10 to 100 μ l) was added to the substrate suspension adjusted to OD 1.0 at 540 nm in 3.0 ml of 0.1 M phosphate buffer, pH 7.0, and monitored the reduction of absorbance at 540 nm.

Reduced and carboxymethylated lysozyme (Cm-lysozyme) was prepared by the method of Crestfield *et al.*¹⁵ Cm-lysozyme was digested with trypsin (1/50, w/w TR-TPCK, Cooper Biomedical Co.) at pH 8.0 and 37°C for 4 h. The tryptic peptides were separated with a reversed-phase (RP) HPLC column (ODS 120A S5, 4.0 × 250 mm, Yamamura Chemical Co., Japan) using the JASCO 800 series HPLC (Japan Spectroscopic Co., Japan). The peptide elution was done with a linear gradient elution system of 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile in solvent A (solvent

B). A gradient of 0% to 50% of solvent B was used for 130 min.

Tryptic peptides were hydrolyzed in evacuated sealed tubes at 110°C for 20 h with constant boiling HCl containing 0.05% β -mercaptoethanol. The resulting hydrolysates were analyzed with an amino acid analyzer (Model 835, Hitachi Co., Japan). The amino acids of tryptic peptides were sequenced by a DABITC/PITC double coupling manual micro sequencing method.^{16,17}

The computing analysis for the protein genealogy of phasianid birds lysozymes was done on the Molecular Evolutionary Analysis System for DNA and Amino Acid Sequences at the National Institute of Genetics, Mishima, Japan. The tree was constructed by the maximum parsimony method.^{18,19}

The HPLC peptide map of CPL was compared with those of other phasianid bird lysozymes. Homologous peptide maps were obtained from CPL and LAPL as shown in Fig. 1. The amino

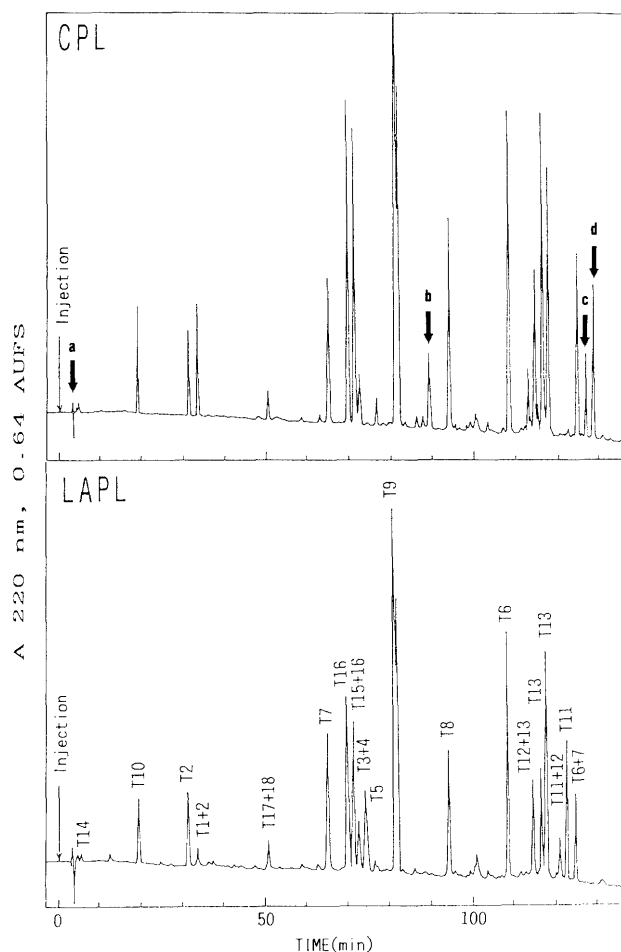


Fig. 1. Comparison of Reversed-phase HPLC Pattern of CPL with That of LAPL.

Peaks in CPL indicated by arrows (peaks a–d) are the peaks appearing in different positions and with different amino acid compositions when compared with LAPL. For detailed conditions of HPLC see the text.

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	-1	10	20	30	40				
CPL	--Y	---	L	NFR	---	F	---	T	
LAPL	--Y	---	L	NYR	---	F	---	T	
KPL	--Y	---	L	NYR	---	F	---	T	
RPL	--Y	---	L	NYR	---	F	---	T	
IPL	--Y	---	L	NYR	---	F	---	T	
TEWL	--Y	---	L	NYR	---	F	---	T	
HEWL	KVFGRC	ELAAAMKR	HGLD	NYRGYS	LGWVCAAK	FESNFNT			
JPL	G	--Y	---	M	NYR	---	F	---	T
QJL	--Y	---	H	KYQ	---	F	---	T	
BQL	--F	---	H	KYR	---	F	---	S	
CQL	--F	---	H	NYR	---	F	---	S	
GHL	--F	---	H	NYR	---	F	---	S	

	50	60	70	80					
CPL	H	---	I	---	R	---	R	N	P
LAPL	H	---	I	---	R	---	R	H	P
KPL	H	---	I	---	R	---	R	H	P
RPL	H	---	I	---	R	---	R	H	S
IPL	H	---	I	---	R	---	R	N	P
TEWL	H	---	I	---	R	---	R	N	P
HEWL	QATNRNTD	GSTDYGI	LQINSRW	CNDGR	TPGSR	NLCNIPC			
JPL	G	---	I	---	R	---	K	H	P
QJL	G	---	I	---	R	---	R	N	P
BQL	Q	---	V	---	R	---	R	N	P
CQL	Q	---	V	---	R	---	R	N	P
GHL	Q	---	V	---	R	---	R	N	P

	90	100	110	120							
CPL	---	L	---	SV	---	V	DGN	---	KR	---	---
LAPL	---	L	---	SV	---	V	DGN	---	NR	---	---
KPL	---	L	---	SV	---	V	DGN	---	NR	---	---
RPL	---	L	---	SV	---	V	DRN	---	NR	---	---
IPL	---	L	---	SV	---	V	DRN	---	NR	---	---
TEWL	---	L	---	SV	---	V	DRN	---	NR	---	---
HEWL	SALLSSDT	IASVNCAKKI	VSDGNC	MNAWVAWR	NRCKG	TDV					
JPL	---	L	---	SV	---	V	DGN	---	KH	---	---
QJL	---	L	---	SV	---	V	DGN	---	NR	---	---
BQL	---	L	---	TV	---	V	DGN	---	NR	---	---
CQL	---	L	---	TV	---	V	DGN	---	NR	---	---
GHL	---	Q	---	TA	---	V	DGN	---	KH	---	---

CPL	NA	TR	---	---
LAPL	NA	TR	---	---
KPL	SV	TR	---	---
RPL	NA	IR	---	---
IPL	HA	IR	---	---
TEWL	HA	IR	---	---
HEWL	QAWIR	GCRL		
JPL	NV	IR	---	---
QJL	NA	IR	---	---
BQL	QA	IR	---	---
CQL	HA	IR	---	---
GHL	RV	IK	---	---

Fig. 2. Amino Acid Sequence of CPL and Its Comparison with Other Phasianid Bird Lysozymes.

Amino acid sequence of HEWL and positions containing substituted amino acid are indicated by single letters. Other positions that contain no substituted amino acids are indicated by dashes.

acid compositions of all peaks of CPL were analyzed. The peptides in each peaks of CPL which have both the identical amino acid composition and the identical elution position to LAPL were considered to have the identical amino acid sequence as reported in our previous paper.¹⁰⁻¹³⁾ On the CPL peptide map, the peaks of T5, T11, and T14 of LAPL were not detected and new peaks a-d (indicated by arrows in the figure) were detected. Peptide a corresponded to peptide T14 of LAPL has a Lys residue instead of Asn. Peptide b corresponded to peptide T5 contained substitution of Tyr→Phe. Peptides c and d corresponded to peptides T11+12 and T11, respectively, contained a substitution of His→Asn, which indicated the substitution in peptide T11. Sequence analysis proved that peptide T5 had Phe at position 6 (substitution of Phe20 for Tyr20). Peptide T11 had Asn at position 4 (substitution of Asn77 for His77). Peptide T14 had the sequence of Lys-Arg (substitution of Lys113 for Asn113). Other peptides of CPL were judged to be identical to those of LAPL by their amino acid compositions and their positions on the map.

The amino acid sequence found for CPL was compared with other phasianid bird lysozymes (Fig. 2). Three amino acid substitutions were at positions 20, 77, and 113 when compared with LAPL sequence and seven substitutions, Tyr3, Leu15, Phe20, His41, Lys113, Asn121, and Thr124 were found when compared with HEWL. These substitutions occupied three of four hyper-variable positions (positions 15, 41, 102, and 121) found in phasianid birds.¹³⁾ The substitution of Phe for Tyr at

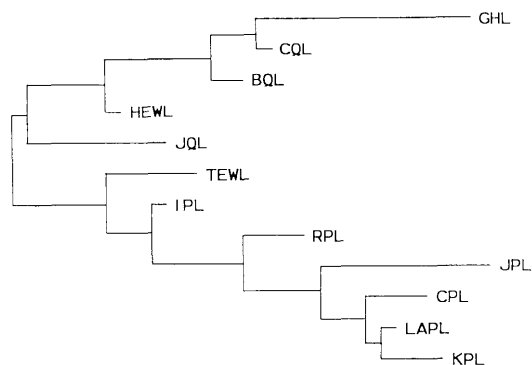


Fig. 3. Protein Genealogy of Phasianid Bird Lysozymes Constructed by Maximum Parsimony Method.

position 20 in CPL was the first finding in bird's lysozyme. Only one substitution (Asn113 to Lys113) was found at subsite F. However, the occurrence of a basic amino acid at this site did not change the activity when chitooligosaccharide was used as a substrate.

The constructed phylogenetic tree of phasianid bird lysozymes is shown in Fig. 3. As described previously, phasianid birds were classified into two groups, the chicken-quail group and the pheasant-peafowl-turkey group, and this classification did not coincide with subfamilies classified by morphological aspects.¹¹⁾

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