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AMINO ACID SEQUENCE OF GUINEA-HEN EGG-WHITE LYSOZYME*

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SUMMARY

Guinea-hen egg-white lysozyme was obtained in a chromatographically pure state by ion-exchange chromatography on Amberlite CG-50. The reduced alkylated enzyme was subjected to digestion with trypsin. The resulting peptides were purified by a combination of Dowex 1-X2 chromatography and electrophoresis and chromatography on paper. The amino acid sequence of these peptides was determined chiefly by the Edman dansylation procedure. Alignment of the tryptic peptides into a single chain containing 129 amino acids was determined from basic overlap peptides obtained by digestion of the enzyme with chymotrypsin. 10 replacements were noted between guinea hen and hen lysozymes. Further comparisons were achieved with 4 other bird egg-white lysozymes the structures of which were already known.

INTRODUCTION

Complete amino acid sequence data on hen^{1,2}, duck II³, quail⁴ and turkey⁵ egg-white lysozymes (mucoprotein *N*-acetylmuramylhydrolase, EC 3.2.1.17) as well as on human milk^{6,7,19} and phage lysozymes⁸ are actually available. In order to extend our comparative studies concerning lysozymes from different origins especially from different bird orders and families, we decided to establish the primary structure of guinea-hen (*Numida meleagris*) egg-white lysozyme.

MATERIAL AND METHODS

Guinea-hen egg-white samples were obtained from the experimental farm of the laboratory of Photobiology, C.N.R.S., Gif-sur-Yvette. Hen egg-white lysozyme, trypsin (EC 3.4.4.4) and chymotrypsin A (EC 3.4.4.5) were purchased from Worthington.

Sephadex G-25 fine and G-75 fine were obtained from Pharmacia and Amberlite CG-50 (Type II) and Dowex 1-X2 (200-400 mesh) from Touzart and Matignon (Paris). All other reagents were obtained from Merck or Prolabo.

* 78th communication on lysozymes. 77th communication: I. BERNIER, E. VAN LEEMPUTTEN, M. HORISBERGER, D. A. BUSH AND P. JOLLÈS, *FEBS Lett.*, 14 (1971) 100.

Lysis of *Micrococcus luteus* (formerly named *Micrococcus lysodeikticus*) was measured according to the procedure of JOLLÈS⁹; the absorbance at 280 nm was used to measure protein.

The purification procedure of guinea-hen egg-white lysozyme utilized was based upon published methods¹⁰. Pooled guinea-hen egg-white (230 ml) was diluted 1:5 in water and the pH was adjusted to 4.5 with 1 M acetic acid. After 1 h, the solution was centrifuged and the formed precipitate was eliminated. 90 ml of Amberlite CG-50, equilibrated in a 0.2 M phosphate buffer (pH 6.5), were added to the clear supernatant and the suspension was stirred at 4° overnight. The rest of the purification (except the dialysis) was carried out at room temperature. The resin, with the lysozyme bound to it, was allowed to settle, and the supernatant, devoid of lytic activity, was discarded. The resin was washed with water and with 0.2 M phosphate buffer (pH 6.5). The lysozyme was eluted with 0.8 M phosphate buffer (pH 6.5). After a short dialysis (1 h) against distilled water at 4°, the biologically active material was subjected to gel filtration on a Sephadex G-75 (120 cm × 2.1 cm) or a Sephadex G-25 (100 cm × 3.3 cm) column, equilibrated with 0.1 M acetic acid. The material in the peak, which included all of the lysozyme activity, was lyophilized. From 230 ml egg white, 414 mg lysozyme (expressed as hen egg-white lysozyme) were obtained. This material was then further purified by ion-exchange chromatography on Amberlite CG-50, desalted on Sephadex G-25 and finally lyophilized.

Starch gel electrophoresis¹¹ was performed at pH 3.8 and at 4° and acrylamide gel electrophoresis at pH 2.9 (90 V, 5 mA, 30 min).

The amino acid composition was established with a Technicon Autoanalyzer after total hydrolysis (6 M HCl at 110° for 18, 48 and 72 h under vacuum). The tryptophan content was determined according to SPIES AND CHAMBERS¹².

Reduction and alkylation

150 mg lysozyme were dissolved in 5 ml of a 0.1 M Tris-HCl buffer (pH 8) containing 8 M urea. 0.75 ml mercaptoethanol was added and the solution was left for 3 h at 38° under N₂; after addition of 1.63 g iodoacetic acid and adjustment of the pH to 8 with NaOH, the mixture was kept at 20° for 20 min. The reduced protein was precipitated with 10 vol. of the mixture acetone-1 M HCl (39:1, v/v). The remaining reagents were eliminated by dialysis against water.

Tryptic hydrolysis

130 mg of reduced lysozyme were digested during 7 h with 1.3 mg trypsin at 37° and pH 8 (0.1 M NH₄HCO₃). Trypsin was pretreated during 16 h with 0.0625 M HCl at 37°.

Separation of the tryptic peptides

The tryptic peptides were chromatographed on a 140 cm × 1.2 cm column of Dowex 1-X2 equilibrated with a solution containing 1% pyridine and 1% collidine, the pH of which had been adjusted to 8.4 with acetic acid. The elution of the most basic peptides occurred at this pH. Gradient elution was then begun by adding successively 0.1 M, 0.5 M, 1 M and 2 M acetic acid into a 300-ml mixing chamber containing buffer solution (pH 8.4) in the presence of 0.1% thiodiglycol; by this procedure, neutral and acidic peptides were eluted. The peptides were characterized by a ninhydrin determination after alkaline hydrolysis. Nearly all the characterized

peaks were further purified by preparative paper chromatography (Whatman No. 1) in the solvents: (A), *n*-butanol–formic acid–water (75:15:10, by vol.) or (B), *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by vol.).

Chymotryptic hydrolysis

120 mg of reduced lysozyme were digested during 24 h with 2.4 mg chymotrypsin at 37° and pH 8 in 0.1 M NH_4HCO_3 .

Separation of the chymotryptic peptides

The chymotryptic peptides dissolved in 75% formic acid were first filtered on a Sephadex G-25 column (200 cm $\times$ 2 cm) with 30% acetic acid as eluent. Eight fractions were collected and submitted to a preparative paper electrophoresis (Whatman No. 1) at pH 6.5 (pyridine–water–acetic acid, 100:900:4, by vol.) and at 50 V/cm.

Determination of the structure of the peptides

The structures were established in detail following the usual methods, chiefly the Edman procedure dansyl method, carboxypeptidase and aminopeptidase digestions; chymotryptic digestion of a tryptic peptide, tryptic digestion of a chymotryptic peptide when possible, partial acid hydrolysis. The different procedures have recently been described⁶ (see also a more general review, ref. 13).

RESULTS

Ion-exchange chromatography and criteria of purity of guinea-hen egg-white lysozyme

Guinea-hen egg-white lysozyme emerged as a single symmetrical protein peak from a column of Amberlite CG-50 equilibrated with a 0.2 M sodium phosphate buffer (pH 7.56) (Fig. 1). The specific activity was constant across the peak. The lysozyme moved as a single protein zone upon starch gel electrophoresis and acrylamide gel electrophoresis under the conditions described in the experimental part.

Specific activity

In its capacity to lyse *M. luteus* under the conditions used, guinea-hen egg-white lysozyme did not differ significantly from the hen egg-white lysozyme.

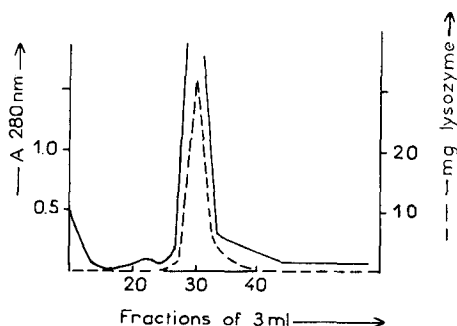


Fig. 1. Ion-exchange chromatography on Amberlite CG-50 (29 cm $\times$ 1.5 cm), pH 7.56, 0.2 M sodium phosphate buffer, of 200 mg purified guinea-hen egg-white lysozyme (quantity expressed as hen egg-white lysozyme).

Molecular size

A single active peak was obtained on Sephadex G-75 at the same point at which hen- and duck II egg-white lysozymes emerged when passed over that column. Thus guinea-hen lysozyme does not seem to differ from hen or duck II lysozymes in molecular size.

Amino acid composition

The amino acid composition was established after different hydrolysis times. Values for serine and threonine were extrapolated to zero time of hydrolysis, whereas those for valine and isoleucine were taken from the 72-h hydrolysate. The composition is indicated in Table I.

TABLE I

AMINO ACID COMPOSITION OF GUINEA-HEN EGG-WHITE LYSOZYME

Amino acid	Residues per mole			
	18 h hydrolysis*	48 h hydrolysis	72 h hydrolysis	Nearest integer
Asp	19.0	17.2	15.9	20**
Thr	6.4	5.3	4.4	7**
Ser	9.4	8.5	8.0	10**
Glu	4.9	4.9	4.7	5
Pro	2.0	2.0	1.9	2
Gly	11.9	11.7	11.7	12
Ala	12.0	11.9	11.8	12
Val	6.4	6.5	6.7	7
(Cys-)	7.5	7.4	7.0	8
Met	1.9	1.7	1.5	2
Ile	4.7	4.7	4.9	5
Leu	6.8	6.8	6.7	7
Tyr	2.9	2.8	2.5	3
Phe	3.0	2.9	3.0	3
Trp	5.8***			6
Lys	7.7	7.7	7.5	8
His	2.0	2.0	1.8	2
Arg	9.6	9.3	9.3	10
Total				129

* Residues per mole calculated on the basis of 2 residues of histidine.

** Value extrapolated to zero time hydrolysis.

*** Characterized following the procedure of SPIES AND CHAMBERS¹².

N- and C-terminal amino acids and sequences

By the Edman degradation procedure combined with the dansylation procedure, Lys-Val-Phe was characterized as the N-terminal sequence of the enzyme. Leucine was the unique amino acid liberated by the action of carboxypeptidase A.

Tryptic peptides

After chromatography of the tryptic hydrolysate on Dowex 1-X2, 14 peaks were characterized (Fig. 2). In Table II, the R_F and mobility (m) values (at pH 6.5), and the yields of 16 tryptic peptides are indicated; these latter account for all the amino acids present in the molecule (Table III). Peak T3 and Peak T6 each contained

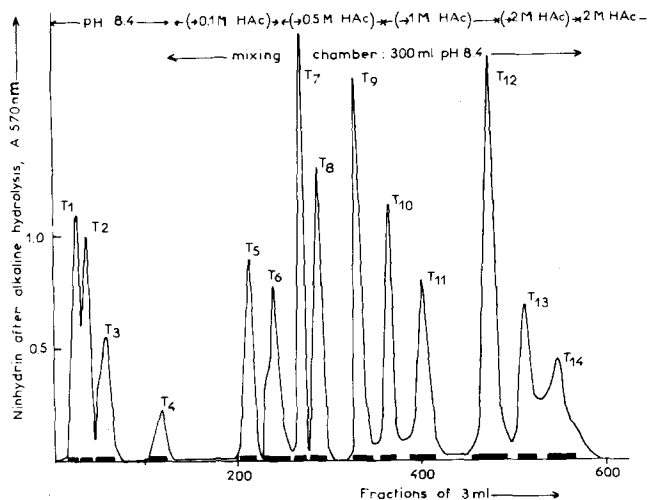


Fig. 2. Chromatography on Dowex 1-X2 (140 cm $\times$ 1.2 cm) of the tryptic hydrolysate of reduced alkylated guinea-hen lysozyme (130 mg protein, 7 h enzymic hydrolysis). HAC, acetic acid.

TABLE II

R_F VALUES (IN SOLVENTS A AND B), MOBILITY m (AT pH 6.5), YIELDS AND CHEMICAL STRUCTURE OF THE TRYPTIC PEPTIDES OF REDUCED ALKYLATED GUINEA-HEN EGG-WHITE LYSOZYME

Peptide	m^*	$R_F(A)$	$R_F(B)$	Yield (%)	Structure**
T1a	+1.1	0.05	0.19	120	Lys
T1b	+1.0	0.08	0.22	57	Arg
T1c	+0.80	0.08	0.50	40	Lys-Val-Phe-Gly-Arg
T2	+0.50	0.05	0.26	90	Thr-Pro-Gly-Ser-Arg
T3a	+0.50	0	0.08	60	Lys-His-SCMCys-Lys
T3b	+0.45	0.30	0.60	43	Val-Phe-Gly-Arg
T4	+0.40	0.40	0.70	47	Val-Trp-Ile-Lys
T5	0	0.08	0.28	75	Gly-Thr-Asp-Val-Arg
T6a	0	0	0.16	27	SCMCys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg
T6b	0	0.20	0.40	56	Gly-SCMCys-Arg-Leu
T7	0	0	0.30	76	Phe-Glu-Ser-Asn-Phe-Asn-Ser-Gln-Ala-Thr-Asn-Arg
T8	+0.10	0.02	0.42	63	His-Gly-Leu-Asp-Asn-Tyr-Arg
T9	-0.22	0.05	0.37	54	SCMCys-Glu-Leu-Ala-Ala-Ala-Met-Lys
T10	-0.17	0	0.25	50	Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Val-Leu-Gln-Ile-Asn-Ser-Arg
T11	0	0.12	0.50	37	Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-SCMCys-Ala-Ala-Lys
T12	-0.25	0.02	0.20	55	Asn-Leu-SCMCys-Asn-Ile-Pro-SCMCys-Ser-Ala-Leu-Gln-Ser-Ser-Asp-Ile-Thr-Ala-Thr-Ala-Asn-SCMCys-Ala-Lys
T13	-0.23	0.10	0.47	57	Trp-Trp-SCMCys-Asn-Asp-Gly-Arg
T14	-0.35	0.02	0.54	45	Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg

* $m = 0$ for Gly; $m = +1$ for Arg; $m = -1$ for CySO_3H .

** SCMCys is S-carboxymethylcysteine.

TABLE III
COMPOSITION OF TRYPTIC PEPTIDES FROM REDUCED ALKYLATED GUINEA-HEN EGG-WHITE LYSOZYME
Residue number in parentheses, nearest integer, based on hydrolysis for 18 h.

Amino acid	Tryptic peptide number*																
	T1a	T1b	T1c	T2	T3a	T4	T5	T6b	T7	T8	T9	T10	T11	T12**	T13	T14**	Total
Asp							0.98 (1)		2.70 (3)	1.78 (2)		3.85 (4)	1.05 (1)	3.60 (4)	1.85 (2)	2.65 (3)	20
Thr				0.75 (1)			1.00 (1)		0.77 (1)			1.90 (2)		1.75 (2)			7
Ser				1.00 (1)					1.62 (2)			1.80 (2)	0.84 (1)	2.75 (3)		1.00 (1)	10
Glu									1.86 (2)		0.90 (1)	0.90 (1)		0.93 (1)			5
Pro				0.93 (1)										0.95 (1)			2
Gly			1.00 (1)	1.00 (1)			1.00 (1)			1.06 (1)		1.95 (2)	2.00 (2)		1.00 (1)	2.10 (2)	12
Ala									1.00 (1)		2.80 (3)		2.19 (2)	3.85 (4)		1.85 (2)	12
Val			0.84 (1)			0.94 (1)	0.90 (1)					0.85 (1)	0.95 (1)			1.60 (2)	7
(Cys-)					0.79 (1)			0.75 (1)			0.83 (1)		0.85 (1)	2.55 (3)	0.84 (1)		8
Met											0.73 (1)					0.60 (1)	2

Ile										0.91 (1)							1.85 (2)	0.80 (1)	5
Leu											0.85 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.97 (2)			7
Tyr												0.83 (1)	0.95 (1)	0.94 (1)					3
Phe																			3
Trp***																			6
Lys																			8
His																			2
Arg																			10
Total	1	1	5	5	4	4	4	5	4	4	12	7	8	16	12	23	7	15	129

* T6a of Table II is missing as it is composed of T9 + T1b. T3b is not mentioned as it is a part of T1c. Free lysine (T1a) obtained with a high yield (120%); Table II) comes partly from T1c (and thus is not counted here) and partly from residue No. 97 (Table VII), as determined by the study of the chymotryptic peptides (Table VI).

** Based on hydrolysis for 72 h.

*** Identified by the Ehrlich reaction.

^c 2 residues determined by the study of the corresponding chymotryptic peptides (Table VI).

two peptides (T3a, T3b, T6a, T6b) which were separated by paper chromatography; in Peak T1, free lysine (T1a) and free arginine (T1b) were characterized beside peptide T1c. The structures of 10 tryptic peptides of guinea-hen egg-white lysozyme, which had the same amino acid composition, R_F values in two solvents and mobility (m) values at pH 6.5 as the corresponding peptides of hen egg-white lysozyme, were not further studied. The 6 remaining tryptic peptides were sequenced in detail, even when only one difference was noted (Table II).

The structures of the short peptides T3a, T4 and T5 were established by the Edman dansyl technique; in peptide T4, the place of tryptophan was characterized by the absence of coloration of the residual peptide by the Ehrlich reagent.

The establishment of the structures of peptides T7 and T12 is indicated in Tables IV and V.

TABLE IV

ESTABLISHMENT OF THE STRUCTURE OF TRYPTIC PEPTIDE T7

Amino acid composition: Asp₃, Thr₁, Ser₂, Glu₂, Ala₁, Phe₂, Arg₁.

<i>Method</i>	<i>Isolated substances</i>
Dansylation	Phe
Edman dansyl	Phe — Glx — Ser — Asx — Phe → → → → →
Aminopeptidase (15 min, 37°)	Phe, Glu, Ser, Asn (47%) (18%) (15%) (15%)
Carboxypeptidases B + A (24 h, 37°)	(Ala, Thr, Asn) — Arg (70%) (70%) (70%) (80%)
Chymotrypsin (6 h, 37°)	{ Glu — Ser — Asn — Phe Asn — Ser — Gln — (Ala, Thr, Asn) — Arg → → → ($m = +0.26$ at pH 6.5)
Chymotryptic peptide from the enzyme (peptide C4 _{C2})	Asn — Ser — Gln — Ala — Thr — Asn → → → → ← ←
Structure	Phe — Glu — Ser — Asn — Phe — Asn — Ser — Gln — Ala — Thr — Asn — Arg

Structure of peptide T10

Amino acid composition: Asp₄, Thr₂, Ser₂, Glu₁, Gly₂, Val₁, Ile₁, Leu₁, Tyr₁ and Arg₁. Asx was characterized as N-terminal amino acid. After chymotryptic digestion (24 h, 37°), four peptides were obtained: No. 1, Asx-(Asx₂, Thr₂, Ser₁, Gly₁)-Tyr; No. 2, (Gly, Val)-Leu; No. 3, (Gly, Val, Leu)-(Asx₁, Ser₁, Glx₁, Ile₁)-Arg; and No. 4, Glx-(Asx₁, Ser₁, Ile₁)-Arg.

Peptide No. 1 was sequenced by the Edman dansyl procedure; its m at pH 6.5 was -0.56 and remained unchanged after the first degradation step; the N-terminal residue was thus Asn and the two other Asx residues were Asp. Peptide No. 2 gave

rise to 100% Leu after digestion during 1 h at 37° by carboxypeptidase A; the N-terminal sequence Gly-Val was determined by the Edman technique. Peptide No. 3 contained peptide No. 2 as N-terminal sequence; a second chymotryptic digestion gave rise to a peptide with a C-terminal Asn residue and to the dipeptide Ser-Arg. Peptide No. 4 had an $m = 0$ at pH 6.5; its N-terminal residue could be determined by the dansyl method only after pretreatment with 1 M NaOH, indicating an N-terminal Gln residue converted to pyrrolidonecarboxylic acid.

The reported results allowed to indicate the following structure of peptide T10: Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Val-Leu-Gln-Ile-Asn-Ser-Arg.

Chymotryptic peptides and alignment of the tryptic peptides by peptide overlap

The chymotryptic digest was submitted to a filtration on Sephadex G-25; eight fractions (C1-C8) were characterized (Fig. 3). The different chymotryptic peptides contained in these fractions were purified by preparative paper electrophoresis and their structures accounting for all the amino acids present in the molecule are indicated in Table VI. The peptides having the same amino acid composition and the same electrophoretic mobility as the corresponding peptides of hen egg-white lysozyme were not studied in detail. The structures of the peptides containing differences were established by the usual methods.

The chymotryptic peptides containing basic amino acids were used in reconstructing the complete amino acid sequence of guinea-hen lysozyme. Comparisons with the well known hen egg-white lysozyme structure made the solution of this problem easier. Evidence for the overlaps between tryptic peptides is indicated on

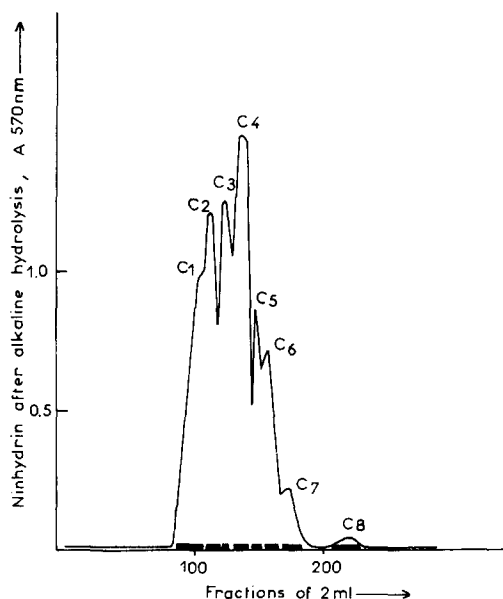


Fig. 3. Filtration on Sephadex G-25 fine (200 cm $\times$ 2 cm), with 30% acetic acid as eluent, of the chymotryptic digest obtained from 120 mg of reduced alkylated guinea-hen lysozyme (24 h enzymic hydrolysis).

TABLE VI

MOBILITY m (AT pH 6.5) VALUES AND STRUCTURES OF THE CHYMOTRYPTIC PEPTIDES CONTAINED IN THE EIGHT FRACTIONS CHARACTERIZED BY FILTRATION ON SEPHADEX G-25 (FIG. 3) AND SEPARATED BY PREPARATIVE PAPER ELECTROPHORESIS AT pH 6.5.

Peptide	m^*	Structure
C1a	+0.41	Ala-Ala-Ala-Met-Lys-Arg-His-Gly-Leu-Asp-Asn-Tyr
C1b	+0.28	Arg-Lys-His-SCMCys-Lys-Gly-Thr-Asp-Val-Arg-Val-Trp
C1c	-0.17	SCMCys-Ala-Lys-Lys-Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp
C2a	+0.93	Ala-Ala-Ala-Met-Lys-Arg-His
C2b	+0.78	Ala-Ala-Ala-Met-Lys-Arg-His-Gly-Leu
C2c	o	SCMCys-Asn-Asp-Gly-Arg-Thr-Pro-Gly-Ser-Arg-Asn-Leu
C3a	+0.35	Ile-Lys-Gly-SCMCys-Arg-Leu
C3b	o	SCMCys-Lys-Gly-Thr-Asp-Val-Arg-Val-Trp
C4a	+1.1	Arg-Lys-His
C4b	+0.56	Lys-Val-Phe
C4c1**	o	Val-SCMCys-Ala-Ala-Lys-Phe
C4c2**	o	Asn-Ser-Gln-Ala-Thr-Asn
C4d	-0.25	Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr
C4e1**	-0.35	Ser-Ser-Asp-Ile-Thr-Ala-Thr-Ala-Asn
C4e2**	-0.35	Gly-Arg-SCMCys-Glu-Leu
C4f	-0.50	SCMCys-Asn-Ile-Pro-SCMCys-Ser-Ala-Leu-Gln
C5a	+0.63	Arg-Gly-Tyr
C5b	o	†PyrGlu-Ile-Asn-Ser-Arg-Trp
C5c	-0.45	Glu-Ser-Asn-Phe
C6a1**	o	Ser-Leu-Gly-Asn-Trp
C6a2**	o	Gly-Val-Leu
C6b	-0.45	Asp-Asn-Tyr
C7a	+0.40	Ser-Arg-Trp
C7b	o	Val-Ala-Trp
C8a	o	Trp

* See Table I.

** Peptides 1 and 2 were separated by preparative paper chromatography in Solvent B.

† PyrGlu; pyrrolidonecarboxylic acid.

Table VII which reports the complete primary structure of guinea-hen egg-white lysozyme.

DISCUSSION

During our previous studies, it was observed that lysozymes from different animal species¹⁴ had different chromatographic behaviours during their ion-exchange chromatography on Amberlite CG-50. For guinea-hen, duck II and duck III lysozymes, the optimum conditions were the same (0.2 M sodium phosphate buffer, pH 7.56); however the elution volumes for the three enzymes were quite different: 160, 180 and 240 ml on a 80 cm × 1 cm column, respectively.

Ten replacements have been noted between guinea-hen and hen egg-white lysozymes and 26 between guinea-hen and duck II lysozymes. This situation explains why a better immunological cross reaction is observed between hen and guinea-hen lysozymes, than between hen and duck II lysozymes¹⁵. However, despite the small number of amino acid replacements between hen and guinea-hen lysozymes, these two enzymes have different behaviours in the presence of their substrates and inhibitors¹⁶. The tryptophan and cystine residues occupy similar positions in all the bird egg-white lysozymes, the structures of which are summarized in Table VII (and

TABLE VII

PRIMARY STRUCTURE OF GUINEA-HEN EGG-WHITE LYSOZYME. COMPARISON WITH HEN^{1,2}, DUCK II³, TURKEY⁵ AND JAPANESE QUAIL⁴ EGG-WHITE LYSOZYMES

		←C4b→	←C4e2→	←C1a→							
		←T1c→	←T6a→								
Guinea-hen (G)	1	Lys	Val-Phe-Gly-Arg	Cys	Glu	Leu-Ala-Ala	Ala	Met-Lys	Arg	His	Gly
Hen (H)											
Duck II (D)			Tyr	Ser							Leu
Turkey (T)			Tyr								Leu
Quail (Q)			Tyr								
				←C5a→	←C6a1→		←C4c1→				
		←T8→		←T11→							
G	17	Leu-Asp-Asn-Tyr-Arg-Gly	Tyr	Ser	Leu-Gly-Asn-Trp	Val	Cys	Ala-Ala			
H											
D											
T											
Q											
			Lys	Gln							
		←C5c→	←C4c2→	←C4d→							
		←T7→									
G	33	Lys-Phe-Glu-Ser-Asn-Phe-Asn	Ser	Gln	Ala-Thr-Asn	Arg-Asn-Thr	Asp	Asn			
H					Thr						
D		Asn	Tyr	Ser	Thr						
T					Thr	His					
Q					Thr						
				←C6a2→	←C5b→	←C8a→					
		←T10→		←T13→							
G	49	Gly-Ser-Thr-Asp-Tyr-Gly-Val	Leu-Gln-Ile-Asn	Ser	Arg-Trp-Trp	Cys					
H			Ile								
D			Ile	Glu							
T			Ile								
Q			Ile								
		←C2c→	←C4f→								
		←T2→	←T12→								
G	65	Asn-Asp-Gly-Arg-Thr-Pro	Gly-Ser-Arg-Asn-Leu	Cys-Asn	Ile-Pro	Cys					
H		Asp	Asn								
D		Asp	Asn	Lys	Lys	Ala	Gly				
T					Lys						
Q											
			←C4e1→								
G	81	Ser-Ala-Leu-Gln-Ser-Ser-Asp	Ile-Thr-Ala-Thr	Ala-Asn	Cys-Ala	Lys					
H			Leu		Ser	Val					
D		Val	Leu	Arg	Glu	Ala	Val	Arg			
T			Leu		Ser	Val					
Q			Leu		Ser	Val					
		←C1c→	←C7b→								
		←T1a→	←T14→								
G	97	Lys-Ile-Val-Ser-Asp-Gly-Asp	Gly-Met-Asn-Ala	Trp-Val	Ala-Trp	Arg					
H											
D		Arg									
T			Ala	Gly							
Q				Val	His						
		←T3a→	←C1b→	←T5→	←T4→	←T6b→					
G	113	Lys-His-Cys-Lys-Gly-Thr-Asp	Val-Arg	Val-Trp	Ile-Lys-Gly	Cys-Arg	Leu				
H		Asn	Arg		Gln	Ala	Arg				
D		Asn	Arg	Arg	Ser	Lys	Arg				
T		Asn	Arg		His	Ala	Arg				
Q		Asn	Arg		Asn	Ala	Arg				

TABLE VIII

NUMBER OF AMINO ACID REPLACEMENTS BETWEEN HEN (H), GUINEA-HEN (G), DUCK II (D II), DUCK III (D III), TURKEY (T), QUAIL (Q) AND HUMAN MILK (Hum) LYSOZYMES*

<i>Lysozyme</i>	<i>Ref.</i>	<i>H</i>	<i>G</i>	<i>D II</i>	<i>D III</i>	<i>T</i>	<i>Q</i>	<i>Hum</i>
H	1	—	10	19	20	7	6	50**
G	Present paper	10	—	26	27	16	15	55**
D II	3	19	26	—	6	19	22	48**
D III	18	20	27	6	—	20	23	47**
T	5	7	16	19	20	—	10	51**
Q	4	6	15	22	23	10	—	52**
Hum	6,7, 19	50**	55**	48**	47**	51**	52**	—

* The changes due to a difference in the nitrogen amide content have not been taken into account in this table.

** With 1 insertion.

also in human milk lysozyme; refs. 6 and 7). The same situation is found for residues glutamic acid (No. 35) and aspartic acid (No. 52) which are essential for the catalytic activity. It is worth mentioning that goose egg-white lysozyme is quite different as it contains only 3 tryptophan and 2 cystine residues¹⁷.

Among the changes between guinea-hen and hen lysozymes, we want to mention more particularly: (a) the appearance of a second histidine residue (No. 114) in a quite basic sequence —Arg–Lys–His– very similar to the sequence containing the other histidine residue (No. 15): —Lys–Arg–His–; (b) the appearance of an additional basic residue, arginine (No. 121), as in human milk lysozyme. Residue No. 121 is particularly exposed to changes in all the studied lysozymes. Furthermore, many replacements occur in the C-terminal sequences (No. 113–125) of the five lysozymes when several other long sequences remain identical (No. 22–32, 104–112 and 42–64 with only one replacement). Table VIII indicates the number of amino acid replacements between the lysozymes mentioned in this study. The changes due to a difference in the nitrogen amide content (Asp, Asn; Glu, Gln) have not been taken into account, as they may occur during the purification of the enzymes or in the course of the chemical structure studies.

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REFERENCES

- 1 J. JOLLÈS, J. JAUREGUI-ADELL, I. BERNIER AND P. JOLLÈS, *Biochim. Biophys. Acta*, 78 (1963) 668.
- 2 R. E. CANFIELD, *J. Biol. Chem.*, 238 (1963) 2698.
- 3 J. HERMANN AND J. JOLLÈS, *Biochim. Biophys. Acta*, 200 (1970) 178.
- 4 M. KANEDA, I. KATO, N. TOMINAGA, K. TITANI AND K. NARITA, *J. Biochem., Tokyo*, 66 (1969) 747.
- 5 J. N. LA RUE AND J. C. SPECK, JR., *J. Biol. Chem.*, 245 (1970) 1985.

- 6 J. JOLLÈS AND P. JOLLÈS, *Helv. Chim. Acta*, 52 (1969) 2671.
- 7 J. JOLLÈS AND P. JOLLÈS, *Helv. Chim. Acta*, 54 (1971) 2668.
- 8 A. TSUGITA AND M. INOUE, *J. Mol. Biol.*, 37 (1968) 201.
- 9 P. JOLLÈS, *Methods Enzymol.*, 5 (1961) 137.
- 10 P. JOLLÈS, H. ZOWALL, J. JAUREGUI-ADELL AND J. JOLLÈS, *J. Chromatogr.*, 8 (1962) 363.
- 11 M. L. GROVES, T. L. McMEEKIN, N. J. HIPPEL AND W. G. GORDON, *Biochim. Biophys. Acta*, 57 (1962) 197.
- 12 J. R. SPIES AND D. C. CHAMBERS, *Anal. Chem.*, 21 (1949) 1249.
- 13 P. JOLLÈS AND J. JOLLÈS, in J. A. V. BUTLER AND D. NOBLE, *Prog. Biophys. and Mol. Biol.*, Vol. 22, Pergamon Press, London, 1971, p. 97.
- 14 P. JOLLÈS, *Angew. Chem. Int. Ed.*, 3 (1964) 28.
- 15 A. FAURE AND P. JOLLÈS, *FEBS Lett.*, 10 (1970) 237.
- 16 J. SAINT-BLANCARD, Y. HENIQUE, D. DUCASSÉ, A.-C. DIANOUX AND P. JOLLÈS, *Bull. Soc. Chim. Biol.*, 50 (1968) 1783.
- 17 A.-C. DIANOUX AND P. JOLLÈS, *Bull. Soc. Chim. Biol.*, 51 (1969) 1559.
- 18 J. HERMANN, J. JOLLÈS AND P. JOLLÈS, *Eur. J. Biochem.*, 24 (1971) 12.
- 19 J. JOLLÈS AND P. JOLLÈS, *FEBS Lett.*, (1972) in the press.

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