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AMINO ACID COMPOSITION OF DUCK AND TURKEY EGG-WHITE LYSOZYMES

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SUMMARY Duck and turkey egg-white lysozymes were purified by CM-cellulose chromatography. Two components of duck lysozyme and a single component of turkey lysozyme were obtained, and the amino acid compositions of these proteins were determined.

Histidine was not detected in either component of duck lysozyme.

INTRODUCTION

Immunochemical studies on hen egg-white lysozyme (HL) have been continued in this laboratory for several years (FUJIO *et al.*, 1959, 1962; SHINKA *et al.*, 1962; SHINKA, 1963; KUWAHARA *et al.*, 1966). The cross reactions between HL and other avian egg-white lysozymes were also studied. The lysozymes compared immunochemically with HL were those from duck (DL) (FUJIO *et al.*, 1962), turkey (TL) and quail (QL) (Imanishi *et al.*, unpublished). For better understanding of the immunochemical differences between these antigens, data on their amino acid compositions followed by sequential studies are important. With regard to HL, much information is available on the amino acid composition and sequence (JOLLÈS *et al.*, 1963, 1964; Canfield, 1963) as well as the tertiary structure (BLAKE *et al.*, 1965). However, as no data have been published on lysozyme from other sources, amino acid analyses of DL and TL were performed.

Furthermore, it has been suggested (FRAENKEL-CONRAT, 1949) that a histidine residue is possibly involved in the active center of HL. Preliminary experiments in this laboratory in collaboration with Dr. K. Narita (IMANISHI *et al.*, 1963) indicated that DL contains no histidine and this casts doubt upon the suspected role of the histidine residue. This finding was confirmed in the present work.

While preparing this paper, the paper of JOLLÈS *et al.* (1965) appeared reporting the amino acid composition of DL. However, a slight difference can be seen between

their data and ours.

MATERIALS AND METHODS

1. *Hen egg-white lysozyme (HL)*

HL crystals were prepared directly from egg-white at its isoelectric point according to the method of ALDERTON and FEVOLD (1946). They were recrystallized three times and purified by chromatography on a CM-cellulose column, and the main component was used. The methods for preparation of DL and TL are described in the " Results."

2. *Amino acid analysis*

A Spinco amino acid analyzer, Model 120B, was used for amino acid analysis. A 5 mg (approximately 0.3 μ mole) sample was dissolved in 1 ml of borate buffer at pH 8.0 and heated at 100°C for 10 minutes and then dried *in vacuo* to remove contaminating ammonia. The procedure for sample hydrolysis and application to the amino acid analyzer, described in the Instruction Manual AIM-2 (Beckman, Spinco Co.) and slightly modified by TSUGITA and FRAENKEL-CONRAT (1962), was followed, except that hydrolysis was carried out at 105°C±1°C.

Tryptophan was calculated from the tyrosine content and the ratio of tyrosine to tryptophan, which could be obtained from the ultraviolet absorption spectra using a Cary 14MP automatic recording spectrophotometer according to the method of GOODWIN and MORTON (1946). Cystine was estimated according to Hirs' method (HIRS, 1956) using performic acid oxidation.

3. *Determination of molecular weight*

The molecular weight was estimated by ultracentrifugation according to Archibald's method (ARCHIBALD, 1947; KLAINER and KEGELES, 1956). For the estimation, 1 per cent solutions of DL and TL in M/50 phosphate buffered saline, pH 6.0, were used. The Archibald runs were carried out at 27,160 rpm and calculations were made with data obtained from the meniscus and assuming the partial specific volume to be 0.7.

RESULTS

1. *Preparation of duck and turkey egg-white lysozymes (DL and TL)*

The preparation method used by FUJIO *et al.* (1962) was used with modifications. DL was first concentrated by adsorption-elution with bentnite (Wako Pure Chemicals) according to the method of ALDERTON *et al.* (1945). Three liters of fresh duck egg-white was homogenized, filtered through gauze and added to an equal volume of 1 per cent bentnite suspension containing 1 per cent KCl. The mixture was stirred for 15 minutes with a homogenizer at room temperature and then centrifuged at 5,000 rpm for 30 minutes in a Servall refrigerated centrifuge. The bentnite was washed three times with one liter of 0.5 M phosphate buffer, pH 7.5, and then three times with one liter of 5 per cent pyridine solution. Then, the DL was eluted with one liter of 5 per cent pyridine solution at pH 5.0 (adjusted with conc. H_2SO_4). The elution was completed within 24 hours. The eluate was dialyzed against distilled water and lyophilized. The dried material was dissolved in phosphate buffered (M/50, pH 6.0) saline and chromatographed on a Sephadex G-75 column with buffered saline. The lysozyme fractions were pooled and lyophilized after dialysis and redissolved in 0.02 M phosphate buffer, pH 8.0. One hundred mg of the material was applied to a CM-cellulose column (1×30 cm) equilibrated with the same buffer and eluted with NaCl in a linear concentration gradient up to 0.4 M all containing 0.02 M phosphate buffer, pH 8.0. The results

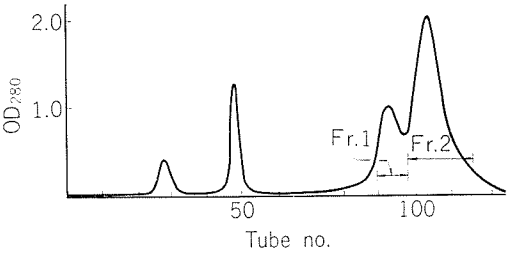

FIGURE 1 Chromatography on CM-cellulose of crude duck lysozyme.
Column size: 1×30 cm; gradient: 0.02 M phosphate buffer, pH 8.0, NaCl molarity changed lineary from 0 to 0.4 M; fraction size: 3.2 ml per tube.
No enzymic activity was found in the first two peaks.

are shown in Fig. 1. The two DL fractions were rechromatographed twice and the homogenous, purified preparations were lyophilized after dialysis. The patterns of rechromatography are shown in Figs. 2 and 3.

TL was concentrated on Amberlite CG-50 according to the method of JOLLÈS *et al.* (1962) and purified by CM-cellulose chromatography. Three hundred ml of homogenized, turkey egg-white was filtered through gauze and mixed with an equal volume of 0.2 M phosphate buffer at pH 6.5. Then 200 ml of Amberlite CG-50 suspension equilibrated with the same buffer was added to the egg-white solution and the mixture was stirred for 4 hours at room temperature. After centrifugation, the sediment was washed twice with the same buffer. The elution was carried out with 300 ml of 0.8 M phosphate buffer at pH 6.5. The elution procedure was repeated twice more. The pooled eluate was dialyzed against distilled water and lyophilized after separation of toluene. One hundred mg of dried material were dissolved in 0.02 M phosphate buffer, pH 5.7, and dialyzed against the same buffer in the cold. The dialyzed solution was applied to a CM-cellulose column (1.5×63 cm) and eluted with a linear gradient of NaCl prepared by mixing 0.02 M phosphate buffer at pH 5.7 with 0.5 M NaCl containing 0.02 M phosphate buffer, pH 8.0. As shown in Fig. 4, only a single peak of TL could be detected. The samples

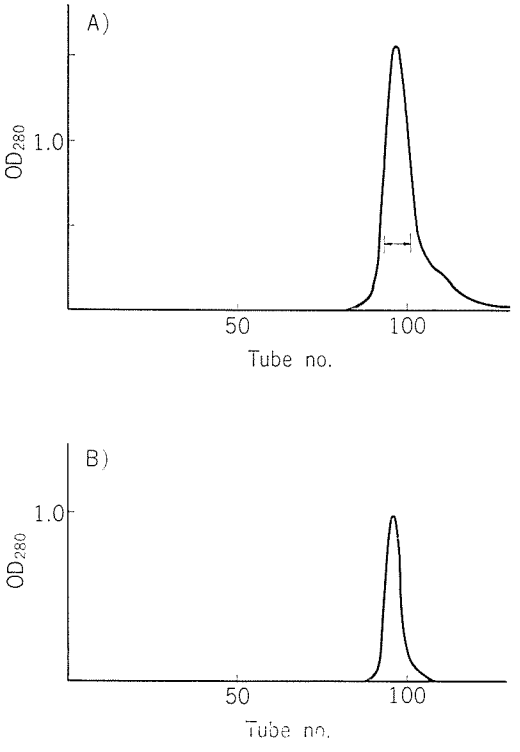

FIGURE 2 A) Rechromatography of DL Fr. 1. B) Second rechromatography of DL Fr. 1. Conditions are given in the legend of Fig. 1.

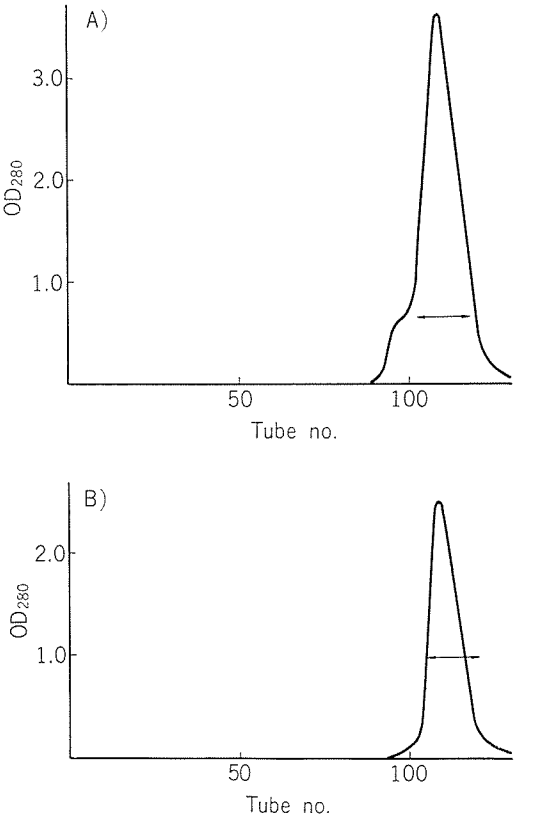

FIGURE 3 A) Rechromatography of DL Fr. 2. B) Second rechromatography of DL Fr. 2. Conditions are given in the legend of Fig. 1.

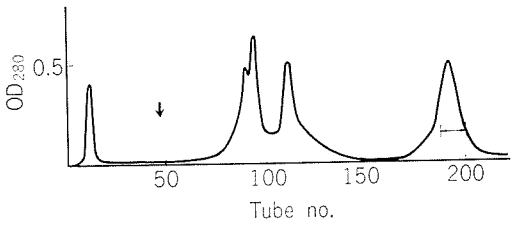

FIGURE 4 Chromatography on CM-cellulose of crude turkey lysozyme.
CM-cellulose column: 1.5×63 cm, 0.02 M phosphate buffer, pH 5.7; gradient to 0.5 M NaCl-0.02 M phosphate buffer, pH 8.0; effluent collected in 11.4 ml fractions; arrow indicates the beginning of gradient elution. The enzymic activity was detected only in the last fraction.

containing TL were pooled, dialyzed in the cold and lyophilized and used for amino acid analysis.

2. *Amino acid composition*

Of the stable neutral amino acids, glycine was chosen as the standard amino acid in the calculation of the amino acid composition because it was present in greatest quantity. Based on a value for the molecular weight of 14,000–15,000, determined by Archibald's method, the most reasonable glycine values were calculated to be 12 moles per mole of DL and 13 moles per mole of TL. The contents of threonine and serine were calculated by ex-

TABLE 1 *Amino acid composition of HL*

(residue/mole after 24 hrs and 72 hrs hydrolysis)

Amino acid	24 hrs		72 hrs		Extrapolated value	Integr. value	Value according to JOLLÈS and CANFIELD
Lys	4.85	4.87	4.75	4.78		5	6
His	0.97	0.97	0.97	1.00		1	1
-$CONH_2$	18.2_1	18.2_1	19.9_3	19.5_8		<18	16
Arg	11.3_2	11.1_0	11.3_2	11.3_3		11	11
Asp	21.0_2	21.1_9	20.9_5	21.2_4		21	21
Thr	6.81	6.86	6.45	6.46	7.03	7	7
Ser	9.44	9.45	8.35	8.38	9.99	10	10
Glu	5.29	5.15	5.25	5.03		5	5
Pro	2.08	2.12	2.24	(2.58)		2	2
Gly	12.00	12.00	12.00	12.00		12	12
Ala	12.0_3	12.0_9	12.0_6	11.9_4		12	12
Cys/2	7.6	—	—	—		8	8
Val	5.62	5.65	6.20	6.30		6	6
Met	2.13	2.09	2.18	2.15		2	2
Ileu	5.54	5.46	5.99	6.09		6	6
Leu	8.17	8.00	8.25	8.37		8	8
Tyr	2.96	2.96	3.02	3.07		3	3
Phe	2.89	2.87	3.07	3.08		3	3
Try	5.1						6
A	1.01	1.13	1.09	1.09			
B	3.12	—	2.95	—			
C	0.54	0.57	0.58	0.59			

trapolating the values obtained from the 24 and 72 hours hydrolysis samples to zero time, while those of valine and isoleucine were calculated from the values after 72 hours hydrolysis only.

As can be seen from Tables 1, 2, 3 and 4, the most remarkable finding was the lack of histidine in both fractions of DL, while HL contained one mole of histidine per mole of HL. On the basis of the results of an iodination experiment, the latter histidine residue has been considered to be involved in the catalytic activity of the enzyme (FRAENKEL-CONRAT, 1949).

There were three components found on amino acid analysis of the hydrolyzates of HL, DL-1, DL-2 and TL, which could not be identified with known amino acids, and were designated as A, B and C. The A and B components were eluted before the lysine peak, in that order, and the C component was eluted between glutamic acid and proline.

The values obtained with HL were in good agreement with those obtained by JOLLÈS *et al.* (1963, 1964) and CANFIELD (1963), except that 5 moles of lysine were detected in the present experiments instead of 6.

DISCUSSION

On the basis of the results reported here, lysozymes from various sources may be compared.

TABLE 2 *Amino acid composition of DL-1*

(residue/mole after 24 hrs and 72 hrs hydrolysis)

Amino acid	24 hrs		72 hrs		Extra-polated value	Integr. value	Difference from HL
Lys	4.88	4.85	5.16	5.16		5	0
His	0	0	0	0		0	−1
-$CONH_2$	18.5_1	17.4_0	15.9_0	16.2_3		<16	(−2)
Arg	13.5_6	13.5_8	13.7_2	13.7_1		14	+3
Asp	19.0_8	18.9_7	18.9_8	18.9_1		19	−2
Thr	6.88	6.90	6.74	6.64	6.99	7	0
Ser	10.3_9	10.3_6	9.72	9.74	10.70	11	+1
Glu	5.17	5.08	5.24	5.15		5	0
Pro	2.00	2.06	2.17	(2.66)		2	0
Gly	12.00	12.00	12.00	12.00		12	0
Ala	11.0_2	11.0_3	10.9_1	11.0_7		11	−1
Cys/2	7.5	—	—	—		8	0
Val	6.48	6.34	7.11	7.07		7	+1
Met	2.11	2.08	2.12	2.08		2	0
Ileu	5.48	5.38	5.98	5.77		6	0
Leu	8.10	7.95	8.32	8.04		8	0
Tyr	4.84	4.77	4.96	4.92		5	+2
Phe	1.07	1.06	0.97	0.93		1	−2
Try	5.8	—	—	—			
A	1.09	1.03	1.06	1.06			
B	1.64	1.59	2.54	2.31			
C	0.47	0.37	0.43	0.46			

TABLE 3 *Amino acid composition of DL-2*

(residue/mole after 24 hrs and 72 hrs hydrolysis)

Amino acid	24 hrs		72 hrs		Extra-polated value	Integr. value	Difference from HL
Lys	4.98	4.93	4.82	4.92		5	0
His	0	0	0	0		0	−1
-$CONH_2$	15.1_3	15.0_2	17.5_0	17.3_7		<15	(−3)
Arg	14.5_0	14.2_0	15.0_8	14.5_9		14	+3
Asp	18.9_7	18.8_2	18.7_8	19.3_8		19	−2
Thr	6.81	6.84	6.63	6.60	6.93	7	0
Ser	9.48	9.49	8.92	8.88	9.78	10	0
Glu	5.11	5.24	5.32	5.05		5	0
Pro	2.50	2.39	2.11	2.16		2	0
Gly	12.00	12.00	12.00	12.00		12	0
Ala	11.0_0	10.9_5	11.0_0	11.0_6		11	−1
Cys/2	7.2	—	—	—		8	0
Val	6.46	6.25	6.96	7.26		7	+1
Met	2.15	2.22	2.02	1.83		2	0
Ileu	5.36	5.32	5.85	5.99		6	0
.Leu	8.00	7.88	8.12	8.29		8	0
Tyr	4.91	4.87	4.51	4.67		5	+2
Phe	0.97	0.94	1.11	1.24		1	−2
Try	5.8	—	—	—			
A	0.81	0.81	0.18	0.24			
B	(3.29)	2.98	1.80	1.97			
C	0.50	0.53	0.61	0.59			

DL-1 and DL-2 appear to differ by one mole of serine and one mole of amide. As the value of serine is an extrapolated one, allowance must be made for about 10 per cent experimental error and since the amide determination has at least this amount of experimental error, observed differences in composition must be regarded as inconclusive.

According to the data from the immunochemical analysis of TL made in this laboratory (IMANISHI *et al.*, unpublished), TL and HL can be regarded as having almost identical behavior in the quantitative precipitin test and in gel-diffusion experiments. Nevertheless, TL apparently differs from HL in several amino acids. The observed differences in the aspartic acid and valine contents we do not consider to be significant since the former may be within the limits of experimental error and the recovery of the latter is influenced by the specific sequence. On the other hand, the differences in lysine, histidine, arginine, glutamic acid, glycine, alanine, leucine, tyrosine and phenylalanine compositions can be assumed to be significant. These differences can be considered to be present in positions not influencing the antigenicity of the protein molecule.

DL differs remarkably from HL in the quantitative precipitin test and in gel-diffusion experiments (FUJIO *et al.*, 1962). It apparently

TABLE 4 *Amino acid composition of TL*

(residue/mole after 24 hrs and 72 hrs hydrolysis)

Amino acid	24 hrs		72 hrs		Extra-polated value	Integr. value	Difference from HL
Lys	5.26	5.30	5.99	5.95		6	+1
His	1.72	1.68	1.91	2.10		2	+1
-$CONH_2$	18.4_1	18.0_0	18.8_1	18.9_2		<18	(0)
Arg	9.80	9.61	10.0_3	9.87		10	−1
Asp	19.7_6	19.9_1	20.3_5	20.1_4		20	−1
Thr	6.76	6.86	6.71	6.73	6.89	7	0
Ser	9.41	9.40	8.94	8.58	9.73	10	0
Glu	3.21	3.18	3.21	3.10		3	−2
Pro	2.10	1.94	2.08	2.17		2	0
Gly	13.00	13.00	13.00	13.00		13	+1
Ala	12.9_2	12.9_4	12.8_2	13.2_9		13	+1
Cys/2	7.2	—	—	—		8	0
Val	4.61	4.58	5.25	5.45		5	−1
Met	2.10	2.10	2.09	2.19		2	0
Ileu	5.78	5.72	6.06	6.15		6	0
Leu	9.09	8.91	9.24	9.42		9	+1
Try	3.66	3.66	3.96	4.07		4	+1
Phe	2.09	2.12	1.95	2.01		2	−1
Try	7.0	—	—	—			
A	0.52	0.58	1.04	1.07			
B	1.92	1.98	2.07	1.76			
C	0.29	0.26	0.25	0.24			

differs in several amino acids. For the reasons given above, the differences in the aspartic acid and valine compositions are not considered to be significant, while the differences in histidine, arginine, alanine, tyrosine and phenylalanine compositions seem to be significant. These differences can be considered to result in differences in the tertiary structure in a sufficiently large portion of the molecules to influence their antigenic characteristics.

JOLLÈS *et al.* (1965) differentiated three peaks of DL by Amberlite CG-50 chromatography. However, only two components were found in our experiments using CM-cellulose chromatography. Comparing the amino acid compositions of the components in their experiment and ours, the first peak of JOLLÈS *et al.* could not be found in our experiments, because their first peak contained one mole of histidine while their second and third peaks as well as DL-1 and DL-2 of our experiments all lacked histidine. The identity of their second peak with DL-1 and third peak with DL-2 cannot be assumed, because the amino acid compositions of each pair differ slightly.

The lower content of lysine (−1 mole) in HL of our experiment, than in the results of JOLLÈS *et al.* (1963, 1964) and CANFIELD (1963), cannot be regarded as an experimental error, and studies are being continued to confirm

this difference.

In regard to the three unknown components (A, B and C) seen on amino acid analysis, it is assumed that they might be either peptides which are resistant to hydrolysis or breakdown products of labile amino acids. However, such components have not been detected in hydrolyzates of tobacco mosaic virus protein or the lysozyme of T_4 phage, using the same methods of analysis.

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