THE OSTRICH (STRUTHIO CAMELUS) EGG-WHITE LYSOZYME.*

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Summary

The purification of Ostrich (*Struthio camelus*) egg-white lysozyme is reported. The quantitative amino acid composition, the molecular weight, the N-terminal sequence (34 amino acids) as well as kinetic studies allow to range this enzyme among the goose type lysozymes.

Introduction

Until recently the vertebrates were known to possess only a simple form of lysozyme (EC 3.2.1.17), exemplified by that found in hen egg-white. This type, designated c (chicken) by PRAGER *et al.*[1], has been characterized at high concentration only in two orders of birds, the Galliformes and the Anseriformes. The amino acid sequences of several of these lysozymes c have been reported[2,3]. Goose egg-white lysozyme discovered by JOLLÈS[4] and CANFIELD[5] has been shown to be radically different from the c type lysozymes on the basis of structural, catalytic and immunological criteria. PRAGER *et al.*[1] detected immunologically this g (goose) type in the egg-white of species representing nine different orders of birds. Only goose egg-white lysozyme has been submitted to extensive studies[2,6–12]; a limited number of results are also available for the black swan enzyme[13]. Thus as the data concerning the g lysozymes are scarce, especially in comparison to the c lysozymes, we decided to report the purification and chemical characterization as well as some enzymatic properties of the lysozyme found in the egg-white of ostrich (*Struthio camelus* from the order of Struthioniformes). Our results allowed this enzyme to be classified among the g type lysozymes.

* 106th communication on lysozymes.

Materials and Methods

Materials

Ostrich eggs were obtained from the "Parc Zoologique de Paris, Bois de Vincennes, France". Hen egg-white lysozyme (six times crystallized) and *Micrococcus luteus* cells were purchased from Miles. Amberlite CG-50 was obtained from Touzart and Matignon (Paris), Sephadex G-25 and G-75 from Pharmacia, CM-cellulose 32 from Whatman. All other reagents (analytical grade) were purchased from Merck or Prolabo, except those employed for the Sequencer which were obtained from Socosi (F-94100 Saint-Maur).

Determination of protein content and of lytic activity

The total proteins were determined by ultraviolet spectrometry at 280 nm using hen lysozyme as a standard. Lytic activity was determined by observing spectrophotometrically at 584 nm the increase in transmittance which occurred during the lysis of a suspension of *Micrococcus luteus* cells in the conditions described by JOLLÈS *et al.*[14].

Determination of molecular weight

The molecular weight was determined by analytical polyacrylamide gel electrophoresis (pH 8.9;

12% acrylamide) in a sodium dodecylsulfate containing system[15]. Carboxypeptidase B, trypsin and hen lysozyme were used as reference substances.

Determination of the composition and of the N-terminal sequence

The methods employed for the determination of the amino acid composition, for the reduction of the protein as well as for the automated Edman degradation in a Socosi Sequencer, Model PS-100, by the Quadrol method were as previously described[16].

Measurement of the apparent affinity constant (Ka, app) for M. luteus cells

The initial velocity of lysis was determined at 20°; pH 6.2; $I = 0.181$ from measurements carried out at 650 nm with a Beckman Acta III spectrophotometer, as already described[8], following the method of LOCQUET *et al.*[17]. It was of particular importance to determine with accuracy the initial velocity of lysis of the substrate by ostrich lysozyme because of its special behaviour in the presence of *M. luteus* cells (see results below). For this purpose low enzyme concentrations (2–5 μg/ml) were used and the substrate concentration was always kept below 450 mg/l because at higher substrate concentrations the lysis occurred too rapidly for measuring the real initial velocity.

Inhibition of lysis by N-acetylglucosamine (GlcNAc)

Lysis of a suspension of *M. luteus* cells (227 mg/l), pH 6,2, $I = 0.164$ by ostrich or hen lysozyme was observed at 584 nm in the presence of various concentrations of GlcNAc in order to determine a possible inhibition effect of this sugar on the lytic activity according to JOLLÈS *et al.*[6].

Purification procedure

Ostrich egg-white (300 ml from a unique egg) was diluted 1:5 in water and mixed during 1 h at 20 °C. The pH was adjusted to 4.5 with 30% acetic acid. After 30 min. at 20°, the solution was filtered and the formed precipitate was discarded. 80 ml of Amberlite CG-50, equilibrated in a 0.1 M ammonium acetate buffer (pH 6.8) were added to the clear supernatant and the suspension was stirred at 20° during 4 h. 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 successively with a 0.1 M and 0.4 M ammonium acetate buffer (pH 6.8). The lysozyme was eluted with 1 M ammonium acetate buffer (pH 6.8). After a short dialysis (1 h) against distilled water, the biologically active material was subjected, after lyophilization, to gel filteration on a Sephadex G-25 column (140 cm × 3.4 cm) equilibrated with 0.1 M acetic acid which was also used as eluent. The active material was concentrated in a desiccator and further purified by ion-exchange chromatography on a CM-cellulose 32 column (12 cm × 2.5 cm) equilibrated with a 0.1 M ammonium acetate solution; gradient elution was then begun by adding a 0.6 M ammonium acetate solution into a 300 ml mixing chamber containing the 0.1 M solution; by this procedure, all the lysozyme activity was recovered in a peak which was dialyzed (1 h) against distilled water, concentrated in a desiccator and finally desalted on a Sephadex G-25 column (140 cm × 3.4 cm) with 0.1 M acetic acid as eluent. The biologically active fractions were concentrated in a desiccator.

Results

Lysozyme content

Five ostrich eggs were used in the course of the present research. They contained each about 700–900 ml egg-white. The lysozyme content of ostrich egg-white was rather variable and reached from 180 mg to 530 mg/kg compared to 4500 ± 900 mg enzyme/kg for hen egg-white. The quantities were calculated from the enzymic activities determined in the usual phosphate buffer (pH 6,2; $I = 0.164$) assuming that both enzymes had the same activity. The activity determination was not sensitive to I (from 0.02 to 0.164).

Purification

Table 1 summarizes the purification procedure achieved with the egg-white of a unique egg and indicates the quantity of active material recovered at each step using 1 kg egg-white as starting material. Figure, 1 illustrates the chromatography on CM-cellulose. The final

Table 1. Summary of the purification procedure of the lysozyme isolated from 1 kg ostrich egg-white.
The "total activity" was expressed as hen lysozyme; experimental conditions: pH 6.2, $I = 0.164$

Step	Total protein (mg)	Total activity (mg)	Specific activity	Yield (%)
1. Dilution of egg-white	33.330	1360	0.040	
2. Treatment at pH 4.5	23.330	916	0.039	67.3
3. Adsorption on Amberlite, elution, dialysis	1.581	885	0.56	65.0
4. Sephadex G-25, concentration	591	784	1.32	57.6
5. CM-cellulose, elution, dialysis	56	300	5.35	22.0
6. Sephadex G-25, concentration	28	298	10.6	21.9

enzyme preparation was purified 265 fold and 28 mg lysozyme (quantity expressed by weight) was obtained from 1 kg of egg-white.

The purity of the enzyme was ascertained (see below) by its electrophoretic (acrylamide gel electrophoresis) and chromatographic behaviour (symmetrical peak on Sephadex G-75), by the constant amino acid composition from three different preparations achieved with three different eggs and especially by the unique N-terminal sequence determined by a Sequencer.

Molecular weight

The final biologically active substance moved as a single protein zone upon acrylamide gel electrophoresis in the presence of sodium dodecylsulfate at pH 8.9; it had a quite different

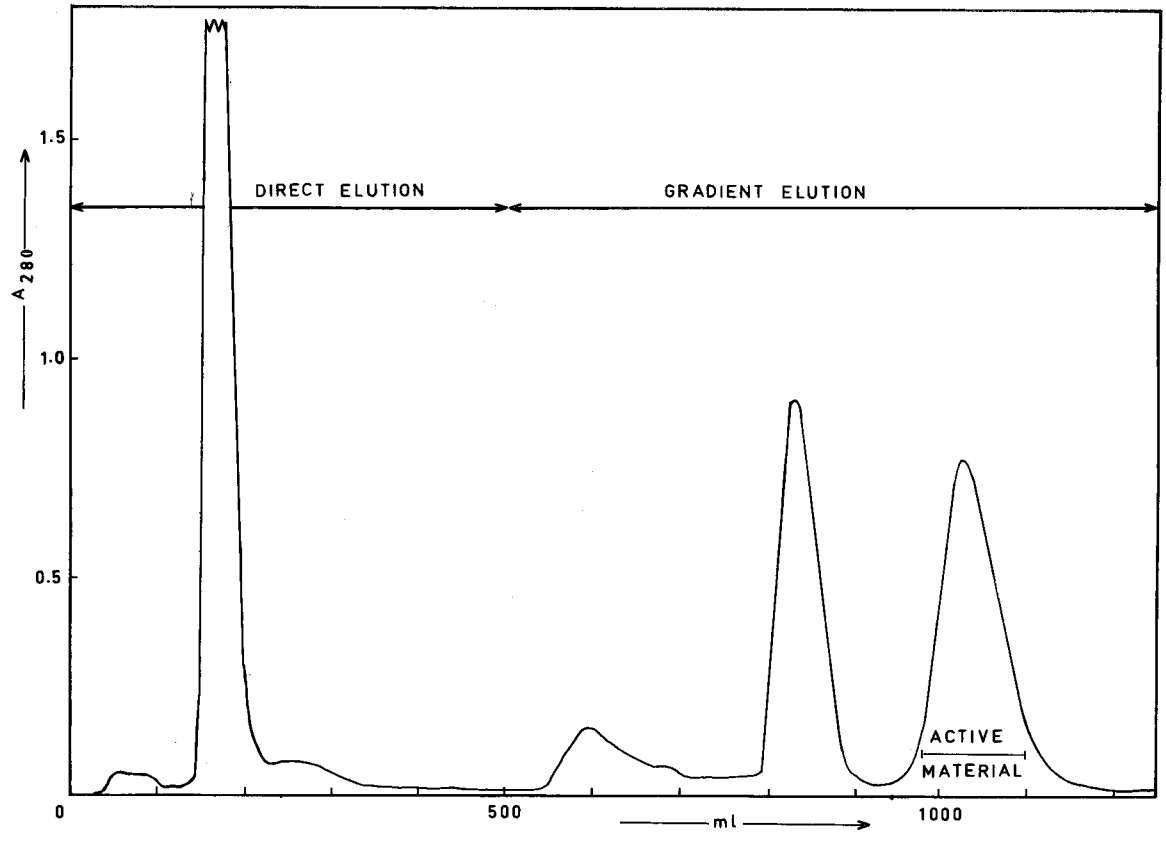

Fig. 1. Chromatography on CM-cellulose 32 (12 cm × 2.5 cm) of 120 mg material recovered after the 4th purification step (Table 1); total activity: 180 mg expressed as hen lysozyme (pH 6.2; $\underline{I} = 0.164$). Direct elution, gradient elution: for details, see "Purification procedure".

behaviour from hen lysozyme. A molecular weight of 20,500 was deduced.

Quantitative amino acid composition

The amino acid composition of ostrich lysozyme is given in Table 2. Three different enzyme preparations obtained from three different eggs gave the same result.

N-terminal sequence

Table 3 indicates the N-terminal sequence established by a Sequencer as well as quantitative figures concerning the characterization of some phenylthiohydantoin-amino acids.

Kinetical data

First the study of the lysis of *M. luteus* cells by

Table 2. Amino acid composition of ostrich lysozyme determined after total hydrolysis (18, 48 and 72 h) of 3 different enzyme preparations. Residues/mole calculated on the basis of 15 alanine residues. Comparison with goose and hen lysozymes (2).

Amino acid	Ostrich				Goose	Hen
	18 h	48 h	72 h	nearest integer		
Asp	21.48	20.40	20.10	22	20	21
Thr	6.35	6.04	5.76	7	13	7
Ser	8.80	8.14	6.89	9	9	10
Glu	14.94	15.15	14.69	15	15	5
Pro	4.74	5.05	4.78	5	5	2
Gly	19.86	20.30	20.00	20	20	12
Ala	15.0	15.0	15.0	15	15	12
Val	9.64	10.07	9.84	10	10	6
(Cys-)	3.83	3.80	2.80	4	4	8
Met	3.84	3.60	1.66	4	3	2
Ile	9.33	10.0	9.94	10	11	6
Leu	8.20	8.64	8.75	9	7	8
Tyr	8.27	7.79	7.17	9	9	3
Phe	2.58	2.45	2.70	3	3	3
Trp (18)				3	3	6
Lys	14.60	14.50	14.37	15	18	6
His	4.60	5.12	5.04	5	5	1
Arg	12.20	12.92	12.56	13	11	11
Total				178	181	129
N-terminal amino acid	Arg				Arg	Lys

Table 3. N-terminal sequence of the ostrich lysozome determined by a Sequencer. Comparison with the partial structures of swan (12) and goose (11) lysozymes: only the replacements were noted. Method of identification were as follows: (a) Phenylthiohydantoin derivative determined by thin layer chromatography; (b) Phenylthiohydantoin derivative determined by gas-liquid chromatography; (c) Amino acid determined with an autoanalyzer after regeneration, results give the percentage. X, unidentified amino acid.

Lysozyme		1								10									
Goose				Asp					Asn					Ile					
Swan				Asp					Asn					Ile					
Ostrich		Arg	Thr	Gly	Cys	Tyr	Gly	Asp	Val	Asn	Arg	Val	Asp	Thr	Thr	Gly	Ala	Ser	
	(a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	(b)		+	20	+	42	12		40	+		32		+	+	8	30	16	
	(c)	15		30	5	33	24	31	36	23	8	27	18			13	15		
Lysozyme				20										30				34	
Goose				Thr					Gly		Ser					← n.d. →			
Swan				Thr	← not determined →														
Ostrich		Cys	Lys	Ser	Ala	Lys	Pro	Glu	Lys	Ile or Leu	Asn	Tyr	Cys	Gly	Val	Ala	X	Ser	
	(a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	
	(b)	+		11	20		3			7	+	7	+	2	6	5		6	
	(c)	2	7		6	9	3	3	3										

ostrich lysozyme was undertaken; two further experiments were then achieved which, previously, allowed the goose lysozyme to be classified among a new type of lysozyme.

Lysis of *M. luteus* cells

Figure 2 presents the increase in transmittance observed at 584 nm of a suspension of *M. luteus* cells (227 mg/l; pH 6.2; $I = 0.164$) by hen (H) and ostrich (O) lysozymes. During the first instants of the reaction, ostrich lysozyme rapidly digested the substrate: then its velocity of lysis decreased significantly in comparison with a quantity of hen lysozyme which attacked the substrate, at the beginning, in a similar manner. An analogous observation was previously reported with goose lysozyme[19]. The high initial velocity of lysis of the ostrich enzyme was in accordance with its high specific activity (10.6, see Table 1; 6 for goose lysozyme).

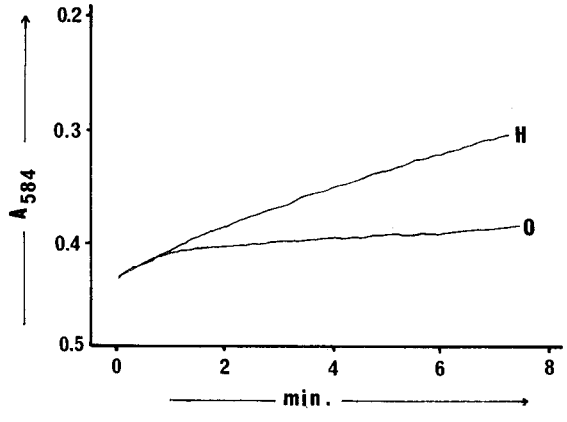

Fig. 2. Kinetics of lysis of a *M. luteus* cells suspension (227 mg/l; pH 6.2; $I = 0.164$) observed at 584 nm by 3 μg of hen (H) and ostrich (O) lysozymes (enzyme quantities expressed as hen egg-white lysozyme equivalents).

Apparent affinity constant for *M. luteus* cells

The graphic representation according to LINEWEAVER and BURK[20] allowed the determination of the apparent affinity constant of ostrich lysozyme for *M. luteus* cells: it was 660 ± 40 mg/l. Under similar experimental conditions, goose and hen lysozymes exhibited Ka,app values of 400 ± 100 and 115 ± 10 mg/l, respectively[17].

Inhibition of the lysis by GlcNAc.

At a concentration as low as 1 μg/ml, ostrich lysozyme was not inhibited by GlcNAc even at a sugar concentration of 7.5×10^{-2} M, a concentration more than sufficient for the inhibition of hen lysozyme.

Discussion

Five radically different types of lysozyme have so far been characterized, namely in hen egg-white ("c", chicken type), in goose egg-white ("g" type), in bacteriophages[21], in plants[22] and in invertebrates[16]. g type lysozymes are much more widespread than the c type enzymes; nevertheless they have hardly been studied at a molecular viewpoint and before the present study only goose egg-white lysozyme has been submitted to an extensive investigation. Chicken and goose lysozymes differ in molecular weight, amino acid composition and more particulary in cystine and trytophan contents, primary structure and enzymic properties, such as optimum pH, and sensitivity towards ionic strength and especially inhibitors such as N-acetylglucosamine (Table 4).

Table 4. Some important differences between c and g type lysozymes.

	Lysozyme			
	c type		g type	
	Hen	Human	Goose	Ostrich
Molecular weight	14,500	14,500	20,500	20,500
Cys content (residues/mole)	8	8	4	4
Trp content (residues/mole)	6	5	3	3
Specific activity	1	3.5±0.5	6±0.5	10.6
Ka,app (mg/l)	115±10	110±10	400±100	660±40
Inhibition by GlcNAc	high	high	almost none	almost none

Ostrich lysozyme has a molecular weight of about 20,000, a low content of cystine and trytophan and a N-terminal sequence nearly related to that of goose lysozyme; it is noteworthy that 3 out of its 4 half-cystine residues are located in this N-terminal moiety. Finally its Ka,app value as well as its insensibility towards N-acetylglucosamine, the classical inhibitor of c type lysozymes, demonstrate clearly that it belongs to the g type lysozymes.

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