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Amino Acid Sequences Containing Cysteine or Cystine Residues in Ovalbumin from Eggs of the Turkey *Melagris gallopavo*

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Abstract

Turkey ovalbumin was isolated from egg white by chromatography on carboxymethylcellulose and further purified after performic acid oxidation by chromatography on DEAE-cellulose in buffer containing 8 M urea. Amino acid analyses and analyses for cysteinyl residues showed three cysteine plus two half-cystine residues are present in turkey ovalbumin.

Five *S*-carboxymethylcysteine-containing peptides from thermolytic digests of ovalbumin which had been reduced and *S*-carboxymethylated with [2-^{14}C]iodoacetic acid were isolated by paper ionophoresis and chromatography and their amino acid sequences determined. The two half-cystine residues involved in the disulfide bond were located by alkylating the cysteine residues with non-radioactive iodoacetic acid, before reducing the disulfide bond and carboxymethylating with [2-^{14}C]-iodoacetic acid. After thermolytic digestion the radioactive peptides were isolated and characterized. Peptides containing half-cystine residues were also identified using a diagonal technique.

Oxidized ovalbumin was digested with thermolysin and pepsin before isolating unbound acidic and bound non-acidic peptides from a sulfonated polystyrene column. The amino acid sequences of the acidic peptides containing cysteic acid or phosphorylated serine residues and the non-acidic peptides containing cysteic acid were determined by the dansyl–Edman method. The acetylated *N*-terminal peptide, which is also present in the acidic fraction, was identified by mass spectrometry.

These amino acid sequence studies have confirmed that turkey ovalbumin contains one cystine and three cysteine residues. The sequences surroudning the half-cystine, cysteine and phosphorylated serine residues as well as the *N*-terminal sequence have been compared with the corresponding sequences in ovalbumins from the hen (*Gallus gallus domesticus*) and the quail (*Coturnix coturnix japonica*).

Introduction

Recent work in our laboratory has explored the relationship of thiol and disulfide to the structure of ovalbumin and of the possibility in proteins like ovalbumin, which contain both thiol and disulfide, of thiol–disulfide interchange leading to erroneous disulfide allocations. In addition we have investigated the amino acid sequences around these residues in ovalbumins from the eggs of birds of various species (Thompson and Fisher 1978*a*; Webster and Thompson 1980; Webster *et al.* 1981; Webster and Thompson 1982). The present study aims to define the number of cysteine and half-cystine residues in ovalbumin from the turkey *Melagris gallopavo* and the amino acid sequences around them.

Smith and Back (1970) showed that the number of cysteine plus half-cystine residues in ovalbumins from various species varies widely and reported nine cysteine plus half-cystine residues for the turkey. Recently Henderson *et al.* (1981) investigated

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the sequences surrounding the two phosphorylated serine residues of ovalbumins of a number of species including the turkey. These phosphorylated sequences are of interest due to their location in the ovalbumin molecule. One of the phosphorylated serine residues (residue 68) occurs in close proximity to one of the half-cystine residues (residue 73) involved in the disulfide bond of ovalbumin from the hen *Gallus gallus domesticus* (Thompson and Fisher 1978*a*; Webster and Thompson 1980) and the other close to the region of the hen ovalbumin molecule which is susceptible to limited proteolysis by subtilisin which results in plakalbumin (Thompson *et al.* 1971). It has been reported by Smith and Back (1970) that the *S*-peptide (a 33-residue *C*-terminal fragment), which is formed during limited proteolysis of hen ovalbumin to give plakalbumin, is not formed in similar experiments with turkey ovalbumin. The *S*-peptide, which is released in acidic 6 M urea-HCl, contains two of the four cysteine residues of hen ovalbumin, and provides, in some species, a convenient way of studying the location and homology of amino acid sequences around these cysteine residues in ovalbumins. The lack of separation of the *S*-peptide was thought by Smith and Back (1970) to be a consequence of an additional disulfide bond in turkey ovalbumin linking it to the plakalbumin protein. Our results do not support this possibility.

Materials and Methods

The methods of cellulose acetate ionophoresis, ultracentrifugation, peptide mapping, amino acid analysis, sulfhydryl group estimation, phosphate analyses, ^{31}P-nuclear magnetic resonance (^{31}P-n.m.r.), cyanogen bromide cleavage, and digestion with thermolysin and pepsin A were substantially the same as previously described (Air and Thompson 1969; Thompson *et al.* 1969; Nash and Thompson 1974; Fisher and Thompson 1979; Webster *et al.* 1981).

The isolation of cysteic acid peptides and other peptides acidic at pH 2, performic acid oxidation and fractionation on sulfonated polystyrene followed the methods previously described (Thompson and Fisher 1978*b*). Blocked *N*-terminal peptides were identified by mass spectrometry (Fisher and Thompson 1979).

High performance liquid chromatography (HPLC) of peptide mixtures was carried out on a Waters High Performance Liquid Chromatograph using a semipreparative, reverse-phase μbondapak C-18 column developed with a linear gradient of 0·1% (v/v) triethylamine–trifluoroacetic acid, pH 2·5, to 70% (v/v) methanol in the starting solution over 60 min. Gradients of 30 min were employed for the fractionation of impure peptides after two-dimensional chromatography using an analytical, reverse-phase μbondapak C-18 column.

Amino Acid Sequence Methods

Digestion with carboxypeptidase A followed the method of Ambler (1972). Amino acid sequences were determined by the dansyl–Edman method (Gray 1967; Hartley 1970) with the following modifications for the Edman procedure.

The peptide (5–100 nmoles) was dissolved in 300 μl 60% (v/v) pyridine–10 μM dithiothreitol. *N*-Ethylmorpholine (10 μl) and phenylisothiocyanate (5 μl) were added under nitrogen and the solution incubated at 55°C for 30 min. Excess reagents were removed by extraction with 1 ml toluene followed by successive extractions with 1 ml heptane–ethyl acetate mixtures (Tarr 1975). The aqueous phase was dried in a stream of nitrogen before cleavage of the phenylthiocarbamyl peptide with 50 μl trifluoroacetic acid under nitrogen at 45°C for 8 min. The trifluoroacetic acid was removed in a stream of nitrogen, 150 μl distilled water added and the phenylthiazolinone extracted with butyl acetate (3 × 1 ml). The aqueous phase was then dried under nitrogen before the next cycle.

Phenylthiazolinones were recovered by drying the butyl acetate extracts under nitrogen and converted to the phenylthiohydantoin derivatives (PTH) with 300 μl 0·1 M HCl at 80°C for 10 min. The PTH's were extracted into ethyl acetate (3 × 500 μl) and the solvent removed under nitrogen before dissolving in methanol for thin-layer chromatography identification (Summers *et al.* 1973; Kulbe 1974).

Preparation of Ovalbumin

Preparation of ovalbumin from turkey eggs commenced within 12 h of laying of the eggs. Crystallization by the method of Warner (1954) of ovalbumin from the isolated egg white was unsuccessful. The egg-white solution containing ovalbumin was recovered after the second precipitation with ammonium sulfate at pH 4·6 which normally results in crystallization. The solution was dialysed against distilled water at 4°C until free of salt and lyophilized. Portions of the lyophilized material were taken up in 0·1 M ammonium acetate, pH 3·6, and dialysed against several changes of the same buffer over 48 h at 4°C, then centrifuged in a Sorvall RC-2B centrifuge at 20000 *g* for 10 min to remove any denatured material. Approximately 2 g of the recovered, dialysed egg white was chromatographed (Webster *et al.* 1981) on a column (10 by 3·4 cm) of carboxymethylcellulose (CMC) equilibrated with the same buffer and eluted with a pH gradient of 0·1 M ammonium acetate buffers from pH 3·6 to pH 5·5 in a linear gradient device (500 ml each chamber). The peak containing ovalbumin, characterized by its mobility on cellulose acetate ionophoresis, was bulked, dialysed against distilled water and lyophilized. This fraction was further characterized by its amino acid composition and sedimentation coefficient in comparison with hen ovalbumin.

The ovalbumin fraction from the CMC chromatography was further purified for amino acid analysis by chromatography of a performic acid-oxidized sample, on a column (2·5 by 14 cm) of DEAE-cellulose utilizing 8 M urea buffers (Thompson and O'Donnell 1966). The lyophilized, oxidized ovalbumin (150-mg lots) was dissolved in the starting buffer, 0·01 M Tris-HCl–1 mM EDTA–8 M urea, pH 8·0, and dialysed to equilibrium. After loading onto the column pre-equilibrated with starting buffer, a salt gradient was developed from 0 to 0·2 M NaCl in the buffer. Fractions were recovered by lyophilization after dialysis against distilled water.

Preparation of Labelled SCM-ovalbumin

The preparation of labelled SCM-ovalbumins and the labelling of disulfide-linked half-cystine residues followed the methods of Webster and Thompson (1980). The denaturing buffers used for disulfide labelling as recommended by Webster and Thompson (1982) were modified by replacing those containing 10 M and 8 M urea with buffers containing 7·5 M and 6 M guanidine-HCl, respectively.

For estimating cysteic acid, which is subject to correction for non-quantitative formation during performic acid oxidation (Moore 1963), the cysteic acid peak was integrated using the leucine standard and corrected by multiplying by 1·08. This factor was based on experiments using hen ovalbumin which is known to have six cysteine plus half-cystine residues.

Diagonal Peptide Mapping

Isolation of thermolytic peptides containing half-cystines involved in the disulfide bond, by diagonal peptide mapping (Brown and Hartley 1966), followed the method of Creighton (1974). Peptides were separated by paper ionophoresis at pH 3·5 (pyridine–acetic acid–water, 1 : 10 : 189 by vol.), oxidized over preformed performic acid vapour for 4 h, dried over NaOH under vacuo, and re-run at pH 3·5 at right angles to the first dimension. Peptides were visualized by staining with 0·1 % (w/v) ninhydrin–1 % (v/v) pyridine in ethanol.

Results

Recrystallization of ovalbumin from turkey egg white was not achieved (cf. Smith and Back 1970), unlike hen ovalbumin (Warner 1954) which crystallizes readily. Turkey egg white was fractionated by gradient elution from CMC by the method of Rhodes *et al.* (1958). After rechromatography under the same conditions the ovalbumin was free from apparent contamination as judged by cellulose acetate electrophoresis. In addition the ovalbumin sedimented as a single peak upon ultracentrifugation. Comparative experiments with hen ovalbumin confirmed similar molecular weights for turkey and hen with $S_{20,w}$ values of 3·27 and 3·35 respectively. Three bands of similar mobility to the phosphorylated ovalbumin forms of ovalbumin from the hen were detected after cellulose acetate electrophoresis. Unlike hen ovalbumin which occurs as diphosphorylated, monophosphorylated and non-phos-

phorylated ovalbumin in the ratio 84 : 14 : 2 (Taborsky 1974) turkey monophosphorylated ovalbumin predominated in the cellulose acetate pattern. Comparative experiments of the phosphate content of hen ovalbumin, which is normally 1·8 residues per mole (Perlmann 1952), was 1·5 residues per mole compared with a phosphate content of 1·1 residues per mole for turkey ovalbumin. Studies by ^{31}P-n.m.r. showed two prominent peaks in the same relative environment as hen ovalbumin, indicating two major phosphorylated sequences in turkey ovalbumin.

Table 1. Amino acid composition of turkey ovalbumin

Samples of turkey ovalbumin or performic acid-oxidized ovalbumin were hydrolysed with 6 M HCl containing 0·1% (w/v) phenol and 0·05% (v/v) mercaptoethanol in sealed evacuated tubes at 110°C for 24 h. Values are given as moles per mole of protein relative to the content of the stable amino acids aspartic acid, proline, glycine, alanine, leucine and phenylalanine which are assumed to total 145 residues per mole. The values for threonine and serine have been corrected 5 and 10% respectively for destruction during hydrolysis. Tyrosine was estimated from ovalbumin hydrolysates. Methionine and cysteine plus half-cystine residues were estimated as methionine sulfone and cysteic acid residues in the performic acid-oxidized ovalbumin, respectively

Amino acid	Mean value	Preferred value	Published value[A]	Hen ovalbumin
Lysine	20·2	20	21	20
Histidine	5·5	6	6	7
Arginine	12·3	12	12	15
Cysteine+half-cystine	5·1	5	9	6
Aspartic acid	31·2	31	31	31
Threonine	21·0	21	19	15
Serine	38·6	39	39	38
Glutamic acid	44·5	45	49	48
Proline	12·2	12	14	14
Glycine	20·7	21	21	19
Alanine	28·8	29	30	35
Valine[B]	22·8	26	28	31
Methionine	16·1	16	14	16
Isoleucine[B]	22·0	26	25	25
Leucine	32·4	32	32	32
Tyrosine	12·2	12	13	10
Phenylalanine	20·3	20	19	20
Tryptophan[C]	4·1	4	4	3
Total		377	386	385

[A] Values taken from Smith and Back (1970).

[B] Values for valine and isoleucine will be low due to incomplete hydrolysis in 24 h. Similar hydrolysates of hen ovalbumin were low by 3·4 and 4·5 residues respectively for approximately the same content of these amino acids, and the preferred values have been corrected up to a similar extent.

[C] Determined by the spectrophotometric method of Beaven and Holiday (1952).

Although the ovalbumin appeared homogeneous on cellulose acetate electrophoresis and five unique cysteine plus half-cystine residues were isolated after two-dimensional separation and autoradiography of a thermolytic digest of [2-^{14}C]*S*-carboxymethyl ovalbumin, some preparations were found, by amino acid analysis,

to be contaminated by a protein of higher cysteine content, as was previously found in the analysis of ovalbumin isolated by similar methods from egg white of the quail (Webster *et al.* 1981).

Analysis of oxidized samples of the main peak from CMC chromatography gave cysteic acid values of 5·2–5·5 residues per mole. No stringent repurification was applied to the samples used for sequence work and no peptides, other than those related to the sequences given in this paper, were found. To establish the analytical value, however, the ovalbumin was rechromatographed on CMC and, after performic acid oxidation, fractionated further on DEAE-cellulose columns using 8 M urea buffers. Table 1 shows the amino acid composition of the performic acid-oxidized ovalbumin recovered from the peak tube on DEAE-urea chromatography. The protein of higher cysteic acid content was in the trailing fractions of the main peak. For the estimation of tyrosine and tryptophan unoxidized ovalbumin was used.

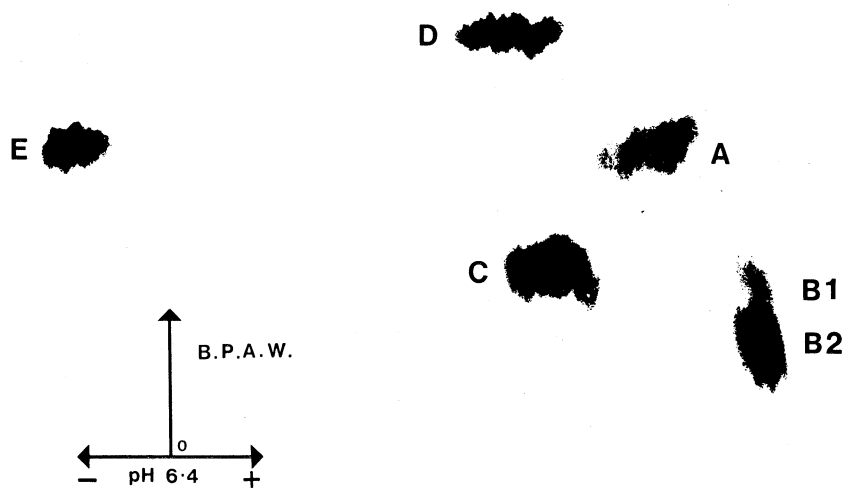


Fig. 1. Autoradiogram of the radioactive zones of [2-^{14}C]carboxymethyl half-cystine plus cysteine thermolytic peptides of turkey ovalbumin after separation by paper ionophoresis, pH 6·4, and paper chromatography with butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 by vol.). The zones are labelled A–E to designate sequences as outlined in the text. The numbered peptides B1, B2 refer to *S*-carboxymethyl and *S*-carboxymethyl sulfoxide forms, respectively, of the same peptide (cf. Webster and Thompson 1980).

The amino acid analysis is similar in total amino acids and the relative proportions of amino acids to hen ovalbumin and to the previously published analysis of turkey ovalbumin by Smith and Back (1970). The high cysteine plus half-cystine content of nine mole residues per mole in the turkey ovalbumin preparation of Smith and Back (1970), compared with our analysis of five cysteine plus half-cystine mole residues per mole of protein, would suggest their sample was contaminated with protein of higher cystine content, even though they purified their ovalbumin by isoelectric focusing. It is possible that the impurity is held by disulfide bonding to the ovalbumin and only released by breaking the disulfide bonds. Nevertheless, it is surprising that larger differences between our analytical values and those of Smith and Back (1970) were not found. It would require approximately 10% (w/w) contamination with ovomucoid to give the value of nine cysteine plus half-cystine residues

reported by Smith and Back (1970), but only 2% (w/w) contamination to give the value of 5·5 obtained for the ovalbumin isolated by CMC chromatography in our experiments.

Separation of the Labelled S-*Carboxymethylcysteine Peptides*

A thermolytic digest of reduced and *S*-carboxymethylated ovalbumin with [2-^{14}C]-iodoacetic acid was fractionated by paper ionophoresis at pH 6·4 and descending paper chromatography. Radioactive peptides were detected by autoradiography (Fig. 1) and further purified by paper ionophoresis at pH 1·9. Five major peptides containing cysteine plus half-cystine residues are clearly indicated, having regard for the presence of the *S*-carboxymethyl and *S*-carboxymethyl sulfoxide forms of the same peptide (Webster and Thompson 1980). They are labelled A–E, in order, from the amino terminal and had the following sequences:

A Phe-CMCys

B Phe-Gly-Asp-SerP-Val-Glu-Ala-Gln-CMCys-Gly-Thr-Ser

C Leu-Gln-CMCys

D Leu-Tyr-CMCys

E Phe-Gly-Arg-CMCys

Residues identified by the dansyl–Edman procedure are underlined by arrows and residues not identified by this method are placed in sequence by homology with hen ovalbumin (McReynolds *et al.* 1978; Thompson and Fisher 1978; Nisbet *et al.* 1981) or the phosphorylated peptides of turkey ovalbumin (Henderson *et al.* 1981). The assignment of amides was done either from the peptide mobility at pH 6·4 (Offord 1966) or by identification of the PTH-amino acid derivative.

Position of the Disulfide Bond

Cysteinyl analysis indicated three cysteine residues (2·5 mole residues per mole protein) and one disulfide bond (5·2 mole residues of cysteine per mole after reduction). Labelling of half-cystine residues of turkey ovalbumin was done by blocking the cysteine residues with non-radioactive iodoacetic acid followed by reduction and *S*-carboxymethylation of the half-cystine residues with [2-^{14}C]iodoacetic acid. This procedure resulted in the specific labelling (greater than 85% of the radioactive label) of the following two half-cystine residues:

B Phe-Gly-Asp-SerP-Val-Glu-Ala-Gln-CMCys-Gly-Thr-Ser

C Leu-Gln-CMCys

Diagonal peptide mapping (Brown and Hartley 1966) was also performed at pH 3·5 to allocate the disulfide bond (Fig. 2). The cysteine residues were *S*-carboxymethylated with non-radioactive iodoacetic acid in the same manner as described above for the preparation of ovalbumin with labelled half-cystine residues. After recovery of the *S*-carboxymethylated protein, the protein was immediately digested

with thermolysin. Peptide was separated by paper ionophoresis at pH 3·5, oxidized, and re-run in the same solvent at right angles to the first dimension.

All peptides which migrated away from the diagonal were analysed. Peptides B and C (Fig. 2) contained cysteic acid, thus confirming the allocation of the disulfide bond by the selective labelling method described above. Peptides A, D and E contained SCM-cysteine sulfone, which has a distinctive migration rate during paper ionophoresis at pH 1·9 and brown colour after staining with ninhydrin, indicating these

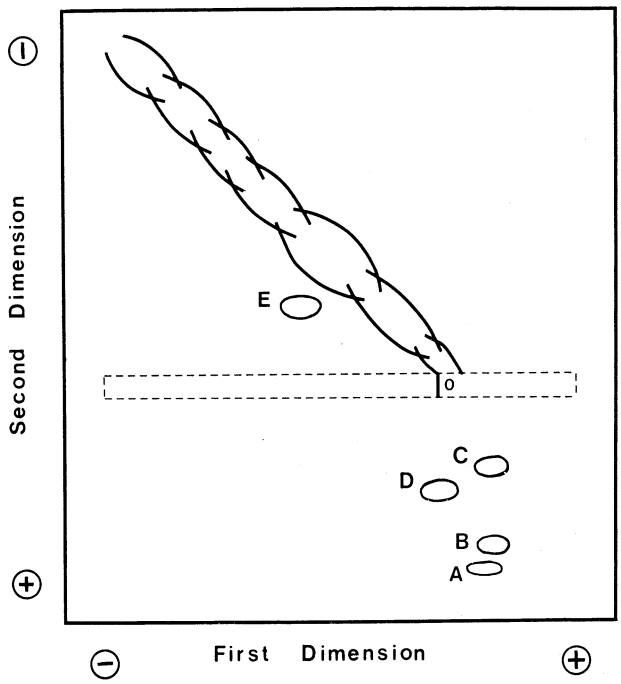


Fig. 2. Diagonal peptide map of a thermolytic digest of turkey ovalbumin which had its cysteine residues *S*-carboxymethylated with iodoacetic acid prior to digestion. The first dimension was run at pH 3·5 then the paper strip was oxidized with performic acid and re-run at right angles to the first dimension. The zones labelled A–E refer to sequences as outlined in the text.

sequences contain cysteine residues in the native molecule. Takahashi (1973) indicated that SCM-cysteine sulfone undergoes extensive decomposition during acid hydrolysis with only 16% being recovered as SCM-cysteine sulfone and the remaining decomposition products yielding no ninhydrin-positive amino acid.

Acidic Peptides in Digests of Oxidized Turkey Ovalbumin

Acidic peptides isolated from peptic, thermolytic and cyanogen bromide digests of performic acid-oxidized or *S*-carboxymethylated ovalbumin not adsorbed on sulfonated polystyrene at acid pH included:

A1	Ac-Gly-Ser	(thermolysin: identified by mass spectrometry)
A2	Ac-Gly-Ser-Ile	(pepsin A)

A3 Ac-Gly-Ser-Ile-Gly-Ala-Val-Ser-Met (CNBr)

A4 Val-Ser-Mes*-Glu-Phe-$CySO_3H$ (thermolysin)

A5 Phe-$CySO_3H$ (thermolysin)

A6 Phe-$CySO_3H$-Phe-Asp-Val (pepsin A)

The thermolytic *N*-terminal-blocked peptide and the CNBr fragment were recovered from *S*-carboxymethylated protein after digestion, elution of the acid non-adsorbed fraction from a sulfonated polystyrene column, and purification by HPLC. The peptic acetylated peptide was similarly purified from a digest of oxidized ovalbumin. The blocked *N*-terminal was identified from the mass spectrum of the thermolytic peptide as an acetyl group and the peptide had the sequence Ac-Gly-Ser.

The overall *N*-terminal sequence of turkey ovalbumin can be deduced to be

Ac-Gly-Ser-Ile-Gly-Ala-Val-Ser-Met-Glu-Phe-Cys-Phe-Asp-Val
1 5 10 14

This sequence is identical to that of hen ovalbumin except a valyl residue has substituted for an alanyl residue at residue 6.

The amino acid analysis of peptides which give the largest overlap are shown in Table 2.

Sequences of the peptic and thermolytic peptides containing one of the half-cystines involved in the disulfide bond and including one of the two phosphorylated serine residues are:

B1 Asp-Lys-Leu-Pro-Gly-Phe-Gly-Asp-SerP-Val-Glu-Ala-Gln-$CySO_3H$-Gly-Thr-Ser (pepsin A)

B2 Phe-Gly-Asp-SerP-Val-Glu-Ala-Gln-$CySO_3H$-Gly-Thr-Ser (thermolysin)

The large peptic peptide was not pure after ionophoresis at pH 6·4 and descending paper chromatography and it was further fractionated by HPLC. This sequence confirms the substitution, found by Henderson *et al.* (1981), of a valyl residue for an isoleucyl residue after the phosphorylated serine in the homologous sequence from hen ovalbumin.

Two additional fragments containing cysteic acid residues were found in the acidic non-adsorbed fraction from the sulfonated polystyrene column. They were:

C1 Leu-Gln-$CySO_3H$ (thermolysin)

D1 Leu-Tyr-$CySO_3H$ (thermolysin)

* Mes, methionine sulfone.

Table 2. Amino acid composition of some peptides from turkey ovalbumin

Hydrolysates were prepared with 6 M HCl for 24 h at 110°C. The values of threonine and serine have been corrected 5 and 10% respectively for losses during hydrolysis. Values are given as moles per mole of peptide, with values for amino acid sequence data in parentheses. The amino acid sequences for the peptides are given in the text

Amino acid	Concn of amino acid (mol/mol peptide) in peptide: A1	A3	A4	A6	B3	C2	D1	D2	E2	P1	M1
Lysine					0·8(1)	0·7(1)		0·9(1)			
Histidine					0·7(1)			1·1(1)			
Arginine									1·0(1)		1·0(1)
Cysteic acid			1·0(1)	1·0(1)	1·0(1)	1·0(1)	1·1(1)	1·0(1)	0·9(1)		
Aspartic acid				1·3(1)	3·3(3)			2·2(2)		1·0(1)	
Threonine					1·0(1)			1·1(1)		0·9(1)	
Serine	0·9(1)	2·1	1·1(1)		3·8(4)			1·2(0)	1·1(1)	1·8(2)	
Glutamic acid			1·0(1)		1·9(2)	3·0(3)				1·4(1)	
Proline					0·8(1)	0·9(1)			1·0(1)		
Glycine	1·0(1)	2·1			2·8(3)				0·9(1)	2·1(2)	2·3(2)
Alanine		1·2			1·0(1)					2·1(2)	
Valine		1·0	0·5(1)	1·0(1)	2·4(3)	0·9(1)				0·8(1)	
Homoserine		0·8									
Methionine sulfone			1·1(1)								
Isoleucine		1·0						1·8(2)	1·0(1)	0·4(1)	
Leucine					1·6(2)	1·0(1)	0·9(1)	1·9(2)			1·5(2)
Tyrosine						1·0(1)	0·7(1)	0·7(1)			0·9(1)
Phenylalanine			0·5(1)	1·7(2)	0·8(1)				0·9(1)		
Total	2	8	6	5	24	9	3	11	7	11	6

A second serine phosphate peptide fragment was also recovered from the acidic, non-adsorbed fraction, the partial sequence of which confirms that obtained by Henderson *et al.* (1981):

P1 Val-Ile-Gly-SerP-Ala-Glu-Ala-Gly-Asp-Ala-Ala-Thr-Ser (thermolysin)

Cysteic Acid-containing Peptides from the Fraction Adsorbed on Sulfonated Polystyrene

The adsorbed fraction of a peptic digest, on a sulfonated polystyrene column, was fractionated, after elution with 1 M NH_3, by HPLC (Fig. 3). Those peak fractions which contained cysteic acid, identified by hydrolysis of portions of each peak and

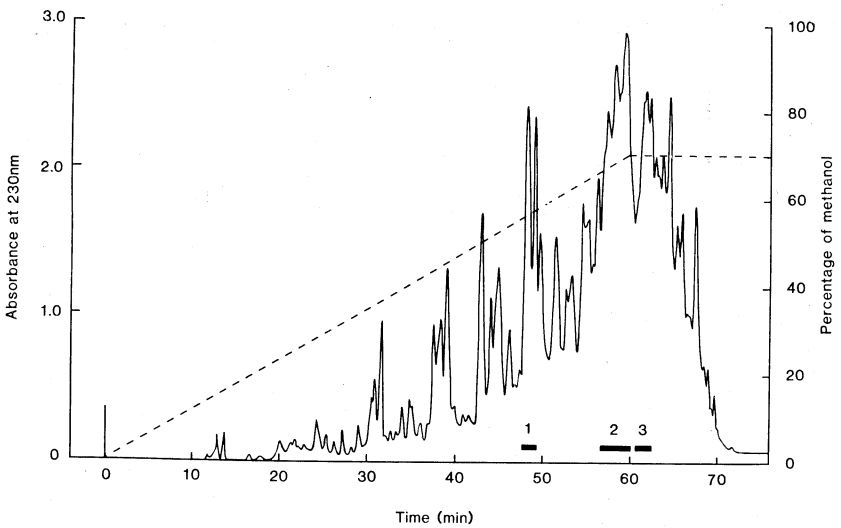


Fig. 3. High performance liquid chromatography of the adsorbed fraction from a sulfonated polystyrene column using 200 nmoles of a peptic digest of oxidized turkey ovalbumin on a semi-preparative μbondapak C-18 column. Gradient elution was from 0·1% (v/v) triethylamine–trifluoroacetic acid, pH 3·0, to 0·1% (v/v) triethylamine–trifluoroacetic acid–70% (v/v) methanol, pH 3·0, over 60 min at a flow rate of 1 ml/min and measured at 230 nm. The areas indicated by bars (1, 2 and 3) were bulked after analysis indicated that they contained cysteic acid. Area 1 contained peptides C2, E2 and M1; area 2 contained peptide E1; area 3 contained peptides D2 and B3.

paper ionophoresis at pH 1·9, were further purified by paper ionophoresis at pH 6·4 and descending paper chromatography. Peptide sequences containing cysteic acid were:

B3 Asp-Lys-Leu-Pro-Gly-Phe-Gly-Asp-SerP-Val-Glu-Ala-
Gln-$CySO_3H$-Gly-Thr-Ser-Val-Asn-Val-His-Ser-Ser-Leu (pepsin A)

C2 Pro-Glu-Tyr-Leu-Gln-$CySO_3H$-Val-Lys-Glu (pepsin A)

D2 Tyr-$CySO_3H$-Ile-Lys-His-Asn-Leu-Thr-Asn-Ile-Leu (pepsin A)

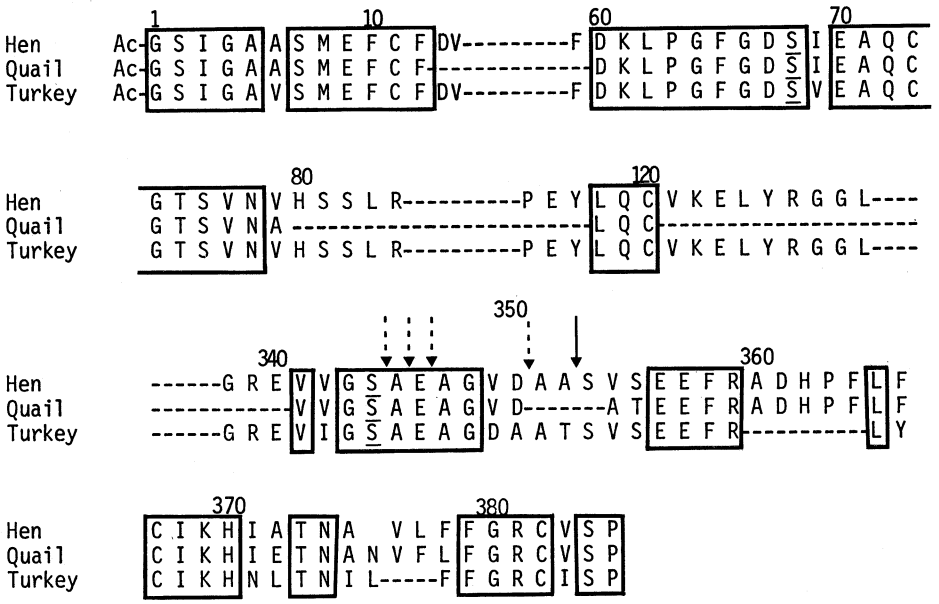
E1 Phe-Phe-Gly-Arg-$CySO_3H$-Ile-Ser-Pro (pepsin A)

E2 Phe-Gly-Arg-$CySO_3H$-Ile-Ser-Pro (pepsin A)

Assignment of arginine and histidine was confirmed from the PTH-amino acid derivative unless excluded by analysis. Sequence C2 extends the area surrounding one of the two half-cystines involved in the disulfide bond. Sequence D2 locates the particular cysteine as the penultimate residue in the ovalbumin sequence. A comparison with the hen ovalbumin sequence (Table 3) shows this region to contain many substitutions (residues 366, 371, 372, 375 and 376). No peptides containing cysteine or half-cystine isolated from turkey ovalbumin were found to have the sequence Phe-Tyr-Cys-Pro-Ile which is found in hen ovalbumin.

Table 3. Comparison of amino acid sequences from ovalbumins of the hen, quail, and turkey

Boxes enclose sequences that are identical. The numbering corresponds to that in hen ovalbumin. Residues from turkey ovalbumin not sequenced by the authors are placed in order from Henderson *et al.* (1981) (59, 84, 338–340 and 354–359). The arrows (↓, ↓) indicate the major and minor bonds, respectively, split in native hen ovalbumin by subtilisin and the dashed lines correspond to unknown regions of sequence in turkey and quail ovalbumins. *S* is phosphoserine. The one-letter code for the amino acids is that recommended by IUPAC-IUB (1968)



Many additional miscellaneous peptides were analysed and sequenced but are not reported in this paper. One peptide, however, isolated from the peptic, non-adsorbed fraction from a sulfonated polystyrene column after HPLC (Fig. 3, area 1) and peptide mapping, lengthened the C sequence. By homology with hen ovalbumin (McReynolds *et al.* 1978; Nisbet *et al.* 1981), this sequence was

M1 Leu-Tyr-Arg-Gly-Gly-Leu

Discussion

Turkey ovalbumin contains three cysteine residues, and also one disulfide bond linking half-cystine residues in identical positions to those found in the hen oval-

bumin (Thompson and Fisher 1978*a*; Webster and Thompson 1980). A comparison of the sequences of hen (McReynolds *et al.* 1978; Thompson and Fisher 1978*a*), quail (Webster *et al.* 1981) and turkey ovalbumin reported in this paper are shown in Table 3.

The homology between the sequences reported here is high with a total of 58 out of 72 amino acid residues identical with hen and quail ovalbumins and 84 out of 97 amino acid residues identical when compared with the hen alone.

Attempts were made to isolate the *S*-peptide of turkey ovalbumin after subtilisin digestion according to the method of Smith (1968). In comparative experiments using hen, quail, duck and emu ovalbumins *S*-peptide was isolated from these species; however, on similar treatment of turkey ovalbumin there was no evidence of any comparable yield of *S*-peptide using cellulose acetate electrophoresis in 6 M urea–formic acid, pH 1·9, for the detection of fragments. This agrees with the report of Smith and Back (1970). Their conclusion, based on a higher cystine content in their sample of ovalbumin, that this is due to an additional disulfide bond linking the plakalbumin protein to the residual *S*-peptide, is not supported by our results. A comparison of the subtilisin-sensitive region (residues 344–354, Table 3) shows some variation about this region. Ottesen (1958) indicated the bond at position 352–353 (Ala-Ser) was the initial site of enzyme action and the arrows in Table 3 indicate where bonds were split subsequently (Thompson *et al.* 1971). Turkey ovalbumin has a substitution at position 352 of a threonyl residue for the alanyl residue. Whether this substitution is sufficient to restrict the limited proteolysis which results in plakalbumin formation is unknown. A number of sequences of this region have been published (Henderson *et al.* 1981) some of which possess the threonyl–alanyl substitution; however, no information was given as to whether these ovalbumins were susceptible to the action of subtilisin.

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References

Air, G. M., and Thompson, E. O. P. (1969). Studies on marsupial proteins. II. Amino acid sequence of the β-chain of haemoglobin from the grey kangaroo, *Macropus giganteus*. *Aust. J. Biol. Sci.* **22**, 1437–54.

Ambler, R. P. (1972). Enzymatic hydrolysis with carboxypeptidases. In 'Methods in Enzymology'. (Eds C. H. W. Hirs and S. N. Timasheff.) Vol. 25. pp. 143–54. (Academic Press: New York.)

Beaven, G. H., and Holiday, E. R. (1952). Ultraviolet absorption spectra of proteins and amino acids. In 'Advances in Protein Chemistry'. (Eds M. L. Anson, K. Bailey and J. T. Edsall.) Vol. 7. pp. 319–86. (Academic Press: New York.)

Brown, J. R., and Hartley, B. S. (1966). Location of disulphide bridges by diagonal paper electrophoresis. The disulphide bridges of bovine chymotrypsinogen A. *Biochem. J.* **101**, 214–28.

Creighton, T. E. (1974). The single-disulphide intermediates in the refolding of reduced pancreatic trypsin inhibitor. *J. Mol. Biol.* **87**, 603–24.

Fisher, W. K., and Thompson, E. O. P. (1979). Myoglobin of the shark *Heterodontus portusjacksoni*: isolation and amino acid sequence. *Aust. J. Biol. Sci.* **32**, 277–94.

Gray, W. R. (1967). Sequential degradation plus dansylation. In 'Methods in Enzymology'. (Ed. C. H. W. Hirs.) Vol. 11. pp. 469–75. (Academic Press: New York.)

Hartley, B. S. (1970). Strategy and tactics in protein chemistry. *Biochem. J.* **119**, 805–22.

Henderson, J. Y., Moir, A. J. G., Fothergill, L. A., and Fothergill, J. E. (1981). Sequences of sixteen phosphoserine peptides from ovalbumins of eight species. *Eur. J. Biochem.* **114**, 439–50.

IUPAC-IUB Commission on Biochemical Nomenclature (CBN) (1968). A one-letter notation for amino acid sequences. Tentative rules. *Eur. J. Biochem.* **5**, 151–3.

Kulbe, K. D. (1974). Micropolyamide thin-layer chromatography of phenylthiohydantoin amino acids (PTH) at subnanomolar level. A rapid micro technique for simultaneous multisample identification after automated Edman degradations. *Anal. Biochem.* **59**, 564–73.

McReynolds, L., O'Malley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Fields, S., Robertson, M., and Brownlee, G. G. (1978). Sequence of chicken ovalbumin mRNA. *Nature* (*London*) **273**, 723–8.

Moore, S. (1963). On the determination of cystine as cysteic acid. *J. Biol. Chem.* **238**, 235–7.

Nash, A. R., and Thompson, E. O. P. (1974). Haemoglobins of the shark, *Heterodontus portusjacksoni*. *Aust. J. Biol. Sci.* **27**, 607–15.

Nisbet, A. D., Saundry, R. H., Moir, A. J. G., Fothergill, L. A., and Fothergill, J. E. (1981). The complete amino-acid sequence of hen ovalbumin. *Eur. J. Biochem.* **115**, 335–45.

Offord, R. E. (1966). Electrophoretic mobilities of peptides on paper and their use in the determination of amide groups. *Nature* (*London*) **211**, 591–3.

Ottesen, M. (1958). The transformation of ovalbumin into plakalbumin. *C. R. Trav. Lab. Carlsberg* (*Ser. Chim.*) **30**, 211–73.

Perlmann, G. E. (1952). Enzymatic dephosphorylation of ovalbumin and plakalbumin. *J. Gen. Physiol.* **35**, 711–26.

Rhodes, M. B., Azari, P. R., and Feeney, R. E. (1958). Analysis, fractionation, and purification of egg white proteins with cellulose-cation exchanger. *J. Biol. Chem.* **230**, 399–408.

Smith, M. B. (1968). The isolation of a large peptide from denatured plakalbumin. *Biochim. Biophys. Acta* **154**, 263–6.

Smith, M. B., and Back, J. F. (1970). Studies on ovalbumin. V. The amino acid composition and some properties of chicken, duck and turkey ovalbumins. *Aust. J. Biol. Sci.* **23**, 1221–7.

Summers, M. R., Smythers, G. W., and Oroszlan, S. (1973). Thin-layer chromatography of subnanomole amounts of phenylthiohydantoin (PTH) amino acids on polyamide sheets. *Anal. Biochem.* **53**, 624–8.

Taborsky, G. (1974). Phosphoproteins. In 'Advances in Protein Chemistry'. (Eds C. B. Anfinsen, J. T. Edsall, F. M. Richards.) Vol. 28. pp. 1–210. (Academic Press: New York.)

Takahashi, K. (1973). Products of performic acid oxidation and acid hydrolysis of *S*-carboxymethylcysteine and related compounds. *J. Biochem.* (*Tokyo*) **74**, 1083–9.

Tarr, G. E. (1975). A general procedure for the manual sequencing of small quantities of peptides. *Anal. Biochem.* **63**, 361–70.

Thompson, E. O. P., and Fisher, W. K. (1978*a*). Amino acid sequences containing half-cystine residues in ovalbumin. *Aust. J. Biol. Sci.* **31**, 433–42.

Thompson, E. O. P., and Fisher, W. K. (1978*b*). A correction and extension of the acetylated amino terminal sequence of ovalbumin. *Aust. J. Biol. Sci.* **31**, 443–6.

Thompson, E. O. P., Hosken, R., and Air, G. M. (1969). Studies on marsupial proteins. I. Polymorphism of haemoglobin of the grey kangaroo *Macropus giganteus*. *Aust. J. Biol. Sci.* **22**, 449–62.

Thompson, E. O. P., and O'Donnell, I. J. (1966). The preparation of the A and B chains from reduced and *S*-carboxymethylated beef insulin. *Aust. J. Biol. Sci.* **19**, 1139–51.

Thompson, E. O. P., Sleigh, R. W., and Smith, M. B. (1971). The amino acid sequence of the large plakalbumin peptide and the C-terminal sequence of ovalbumin. *Aust. J. Biol. Sci.* **24**, 525–34.

Warner, R. C. (1954). Egg proteins. In 'The Proteins'. (Eds H. Neurath and K. Bailey.) Vol. 2A. pp. 435–85. (Academic Press: New York.)

Webster, D. M., and Thompson, E. O. P. (1980). Position of the disulfide bond in ovalbumins of differing heat stability. Elimination of thiol-disulfide interchange as a mechanism for the formation of the ovalbumins. *Aust. J. Biol. Sci.* **33**, 269–78.

Webster, D. M., Fisher, W. K., Koureas, D. D., and Thompson, E. O. P. (1981). Amino acid sequences containing cysteine or cystine residues in ovalbumin from eggs of the quail *Coturnix coturnix japonica*. *Aust. J. Biol. Sci.* **34**, 505–14.

Webster, D. M., and Thompson, E. O. P. (1982). Carboxymethylation of thiol groups in ovalbumin: Implications for proteins that contain both thiol and disulfide groups. *Aust. J. Biol. Sci.* **35**, 125–35.

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