CHEMICAL CHARACTERIZATION OF A NON-COVALENTLY LINKED PEPTIDE FROM PLAKALBUMIN

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Summary

A 33-residue peptide isolated from plakalbumin by dissociation with urea at pH 3 is shown to be derived from the *C*-terminal portion of ovalbumin. The yield of proline obtained on hydrazinolysis was the same as that from ovalbumin, whereas proline was not liberated from the protein component. The *N*-terminal sequence of this peptide was determined by the Edman degradative procedure to be Ser.Val.Ser.Glu.Glu.Phe.Arg.Ala.Asp.

The protein component of dissociated plakalbumin had glutamic acid as its major *C*-terminal residue with significant amounts of alanine, serine, and aspartic acid. The results are consistent with previous reports in the literature regarding the mode of attack of subtilopeptidase A on ovalbumin, the large peptide being bound by non-covalent forces owing to its high content of amino acids with non-polar side chains.

I. Introduction

The conversion of ovalbumin to plakalbumin by mild proteolysis with subtilopeptidase A (EC 3.4.4.16) was shown by Ottesen and coworkers (Ottesen 1958) to involve the initial cleavage of an alanylseryl linkage (cf. Satake, Kurioka, and Neyasaki 1961; Satake, Sasakawa, and Honda 1965) followed by the release of several small peptides.

Recent work by one of us (Smith 1968) has shown that a peptide of molecular weight 3800, with an *N*-terminal serine residue, is firmly bound to the plakalbumin by non-covalent forces and can be isolated and purified by gel filtration in 6M urea solutions at pH 3.

The *N*-terminal sequence and *C*-terminal residue of this peptide has now been established and its location in the ovalbumin molecule can be deduced from these results and from the *C*-terminal residues of the plakalbumin protein component.

II. Materials and Methods

(*a*) *Preparation of Proteins*

Ovalbumin was prepared from fresh eggs by the method of Sørensen and Høyrup (1915–17) and recrystallized four times from $(NH_4)_2SO_4$ solution.

Plakalbumin was prepared by digestion of ovalbumin with subtilopeptidase A (batch 61–3, kindly donated by Professor M. Ottesen) for 60 min at 30°C under the conditions already described (Smith 1968).

After carboxymethylation the plakalbumin peptide and plakalbumin protein components were separated by gel filtration in 6M urea at pH 3 as described by Smith (1968).

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(*b*) *Amino Acid Analyses*

Amino acid analyses were carried out by the accelerated procedure of Spackman (1967) using a Beckman–Spinco model 120C amino acid analyser with a range card (Hamilton 1963) fitted to the analyser when analysing amounts of material of the order of 0·01 μmoles.

(*c*) *Edman Degradations*

Degradations were carried out following the procedure of Blombäck *et al.* (1966) after coupling in 60% pyridine–water containing 6% *N*-ethylmorpholine.

A portion of the ethyl acetate extract (1 ml total) of phenylthiohydantoins was used for thin-layer chromatographic identification of the amino acid derivatives (Cherbuliez, Baehler, and Rabinowitz 1964) whilst a further portion was dried and regenerated to amino acids by heating with 6N HCl at 150°C for 24 hr (Van Orden and Carpenter 1964). An aliquot of the redissolved peptide was withdrawn for amino acid analysis after the first, third, sixth, eighth, and ninth steps to confirm losses of amino acid residues identified by the direct procedure.

(*d*) *Hydrazinolysis*

Various modifications of the hydrazinolysis procedure of Akabori, Ohno, and Narita (1952) were used. Hydrazine sulphate (Bradbury 1958) and Bio-rex 70 (Braun and Schroeder 1967) were added as catalysts in some experiments. The protein or peptide (0·5 μmoles) and catalyst, e.g. 60 mg Bio-rex 70, were weighed into a Pyrex test tube which was then constricted. The material was dried by heating for 24 hr in vacuum in an Abderhalden pistol at 78°C. Anhydrous hydrazine (0·5 ml, 97%; Matheson, Coleman, & Bell Co.) was added by a drawn-out funnel through the constriction, and the tube sealed under vacuum.

The tubes were left (48–96 hr) in an 80°C oven. After reaction, the tubes were opened and carefully lyophilized to remove excess hydrazine. The residue was dissolved in water, transferred to a 5- by 1-cm Bio-rex 70 column, and the amino acids eluted with approximately 30 ml of water. The eluate was lyophilized, dissolved in 3 ml of pH 2·2 buffer, passed through a Millipore filter, and a sample applied to the long column of the amino acid analyser.

This method of separating amino acids from the hydrazides (Blackburn and Lee 1954; Silman, Cebra, and Givol 1962) will not detect *C*-terminal residues of arginine, histidine, or lysine, which require special procedures (Braun and Schroeder 1967).

III. Results

(*a*) N-*Terminal Sequence*

The amino acid analyses of the peptide before and after the repeated application of the phenylisothiocyanate degradative procedure are shown in Table 1. The amino acid composition of the peptide is in agreement with that given by Smith (1968) although some experimental difficulties caused low values for *S*-carboxymethylcysteine.

The phenylthiohydantoins identified after each cycle of degradative reactions are shown in Table 2.

Neither the direct analysis of the phenylthiohydantoins, which were not obtained in the high yields expected from the results of Blombäck *et al.* (1966), nor subtractive amino acid analysis, which on the unpurified material after each degradative step did not always show complete removal of a terminal residue, gave conclusive results by themselves.

However, the combined results (Tables 1 and 2) suggest the terminal sequence Ser.Val.Ser.Glu.Glu.Phe.Arg.Ala.Asp–. The allocation of aspartic acid to residue 9 rather than phenylalanine is based on the thin-layer chromatography since the

TABLE 1

AMINO ACID COMPOSITION OF PLAKALBUMIN PEPTIDE AFTER SUCCESSIVE EDMAN DEGRADATIONS

Amino Acid	Number of Degradative Cycles*						
	None		1	3	6	8†	9
Lysine	0·9	1·0	0·5	0·4	0·8	1·1	0·7
Histidine	1·8	1·9	1·8	1·9	1·8	1·8	1·4
Arginine	1·8	2·0	1·7	1·8	1·8	*1·1*	1·3
SCM-cysteine‡	1·4	2·0	1·6	1·8	1·5	1·0	1·2
Aspartic acid	2·1	2·1	2·1	2·1	2·0	2·0	*1·4*
Threonine§	0·8	1·0	0·9	1·0	0·9	lost	1·0
Serine§	2·7	2·6	*1·9*	*1·2*	1·2	lost	1·0
Glutamic acid	2·2	2·0	2·1	1·5	0·7	0·8	0·8
Proline	2·0	1·9	2·0	1·8	1·9	1·7	1·7
Glycine	1·1	1·0	1·1	1·1	1·1	1·2	1·2
Alanine	3·2	3·0	2·8	3·1	2·8	*2·1*	1·8
Valine	3·0	3·0	3·0	*2·2*	2·1	2·0	1·8
Isoleucine	2·1	1·9	1·9	2·0	1·9	1·9	1·8
Leucine	2·1	2·0	2·0	2·0	2·0	2·0	2·0
Phenylalanine	5·1	5·2	4·9	5·0	*3·9*	4·1	3·3

* The amino acids decreasing in amount and identified as phenylthiohydantoins are given in italics.

† This analysis was from a separate experiment.

‡ *S*-carboxymethylcysteine; some conversion to cysteic acid was observed due to incomplete removal of oxygen.

§ Not corrected for decomposition.

TABLE 2

PHENYLTHIOHYDANTOINS (PTH) IDENTIFIED IN SUCCESSIVE DEGRADATIONS OF PLAKALBUMIN PEPTIDE

Step No.	PTH Identified by Thin-layer Chromatography	Amino Acid Regenerated
1	Serine	Nil*
2	Valine or threonine	Valine
3	Serine	Nil*
4	Glutamic acid	Glutamic acid
5	Glutamic acid	Glutamic acid
6	Phenylalanine	Phenylalanine
7	Arginine	†
8	Alanine	Alanine
9	Aspartic acid or *S*-carboxymethylcysteine	—

* PTH-serine gives extremely poor yields of serine on acid hydrolysis.

† The aqueous phase was not investigated by regeneration.

subtractive amino acid analysis was equivocal. The identifications of subsequent residues became less certain and further degradative cycles were abandoned.

With whole plakalbumin the amino acids serine, glutamic acid, alanine, and valine were qualitatively identified as *N*-terminal residues. After purification of the protein component of plakalbumin only trace amounts of serine, glutamic acid, and alanine were found which seemed more indicative of impurities than of additional cleavage points with subtilopeptidase A.

(*b*) C-*Terminal Residues*

The free amino acids determined in hydrazinolysates of lysozyme, ovalbumin, plakalbumin, plakalbumin peptide, and plakalbumin protein are shown in Table 3. There was considerable variation in the quantitative results obtained using different conditions of catalysis. As Braun and Schroeder (1967) have shown, there is a variation in yield depending on the time and temperature of reaction. In our experiments no study of these variables was made, a temperature of 80°C with heating for 48 hr being used. Under these conditions lysozyme in the presence of 60 mg of Bio-rex 70 as catalyst gave 79% yield of leucine which was less than the 94–96% reported by Braun and Schroeder (1967) after 15–20 hr at 80°C. Ovalbumin gave 34–43% yield of proline and a yield of this order was also obtained from whole plakalbumin and the plakalbumin peptide. In contrast, no proline was liberated from the plakalbumin protein.

Only small amounts of serine, glycine, and alanine were obtained along with proline in hydrazinolysates of the plakalbumin peptide. These are probably artefacts of the procedure, as Braun and Schroeder (1967) have reported that all proteins studied by them gave glycine, serine, and alanine as background but never more than 5% of one residue per mole.

The plakalbumin protein gave predominantly glutamic acid and serine as *C*-terminal residues with the possibility that alanine is also present in significant amounts. As would be anticipated, whole plakalbumin gave the pattern expected from the separate results on plakalbumin protein and peptide.

IV. Discussion

The binding of peptides to proteins by non-covalent forces is well illustrated in the recent work on ribonuclease following mild proteolysis by subtilopeptidase A or subtilisin-BPN′ (Richards and Vithayathil 1960; Doscher and Hirs 1967; Gross and Witkop 1967).

The peptide formed during the conversion of ovalbumin to plakalbumin (Table 1) is rich in amino acids with non-polar side chains. Those amino acids identified in the *N*-terminal sequence point to an even higher concentration of non-polar residues in the unmapped regions of the peptide. The significance of this hydrophobic region to the strong non-covalent bonding of the peptide to the residual protein has yet to be investigated.

The results reported in this paper are consistent with the initial rapid hydrolysis of an alanylseryl bond, as described by various workers, with subsequent release of small peptides. Among the peptides which have been identified are Ala.Ala,

TABLE 3

YIELDS OF *C*-TERMINAL AMINO ACIDS AFTER HYDRAZINOLYSIS

Values are moles/100 moles of peptide or protein, uncorrected for losses. Time of heating, 48 hr at 80°C

Amino Acid	Lysozyme (Bio-rex 70)	Ovalbumin (Bio-rex 70)	Ovalbumin (uncat-alysed)	Plakalbumin Peptide (hydrazine sulphate)	Whole Plakalbumin			Plakalbumin Protein			
					Uncat-alysed	Hydrazine Sulphate	Bio-rex 70 *in vacuo**	Uncat-alysed	Uncat-alysed	Bio-rex 70	Bio-rex 70 *in vacuo*
SCM-cysteine†						16					
Aspartic acid	trace	trace			22·8	14·4		trace	15·2	trace	
Threonine					trace	3	4·1		trace		
Serine		trace		9·5	13·0	9·55	7·1	22·4	18	41·5	7·7
Glutamic acid	trace	4·8	2·7		32·4			60	51	50	24
Proline		34·4	43·3	37·0	59	32·7	27·7				
Glycine	trace	5·3	2·2	6·2	13·4	8·4	4·7	trace	7·9	trace	5·3
Alanine		5·1	1·8	5·0	25	12·3	9·6	trace	7·0	trace	21·8
Valine											
Methionine					trace		2	trace			
Isoleucine					trace			trace			
Leucine	79				trace		2·7				
Phenyl-alanine							6·3				

* 96 hr heating. † *S*-carboxymethylcysteine.

Ala.Gly.Val.Asp, Glu.Ala.Gly.Val.Asp, and Glu.Ala.Gly.Val.Asp.Ala.Ala (Ottesen 1958; Satake, Kurioka, and Neyasaki 1961; Satake, Sasakawa, and Honda 1965). Most of these peptides would be expected to be lost during the dialysis used in the present work in the isolation of plakalbumin and should certainly have been removed from the plakalbumin protein component during gel filtration in 6M urea.

The large plakalbumin peptide of 33 residues with *N*-terminal serine residue and *C*-terminal proline residue is clearly from the *C*-terminal portion of ovalbumin, since the pattern and yield of free amino acids on hydrazinolysis, with proline as the major product, is essentially the same as that obtained with ovalbumin and different from that given by the plakalbumin protein component.

In whole plakalbumin the presence of several *C*-terminal amino acids could be due to peptides bound to the plakalbumin protein. These peptides could arise from small amounts of denatured ovalbumin present during the digestion with subtilopeptidase. The proline is from the large plakalbumin peptide and some aspartic acid and alanine could represent *C*-terminal residues of the smaller peptides released during the proteolytic conversion, with glutamic acid and serine the *C*-terminal residues of the plakalbumin protein. However, these *C*-terminal residues remain in the plakalbumin protein which has been purified to dissociate and remove lower molecular weight peptides. It must be concluded therefore that there are several points of proteolytic cleavage by subtilopeptidase A in the native ovalbumin with glutamic acid and serine as the major *C*-terminal residues. The smaller amounts of alanine, aspartic acid, and glycine are significantly above the level of background amino acids obtained with lysozyme and ovalbumin, and must represent either additional cleavage positions, or the proportion of the smaller peptides, listed above, not quantitatively liberated during the limited proteolysis.

It has become apparent during detailed investigations of other examples of limited proteolysis on native proteins that seldom is there a single point of hydrolytic cleavage. In the case of chymotrypsinogen (Dixon and Webb 1964), trypsinogen (Maroux, Rovery, and Desnuelle 1967), ribonuclease (Doscher and Hirs 1967; Gross and Witkop 1967), pepsinogen (Rajagopolan, Moore, and Stein 1966), and streptococcal proteinase (Liu and Elliott 1965), for example, several sites of peptide-bond cleavage resulting in active products have been established.

For the limited digestion of ovalbumin, Satake, Kurioka, and Tsuzukida (1964) have reported variation in the points of enzyme attack depending on the conditions of digestion. Their interpretation is supported by the present results and a summary of the probable course of the proteolysis by subtilopeptidase A is given in Figure 1. The *N*-acetylGly.Ser.Gly.Ile.Ala– and *C*-terminal –Val.Ser.Pro sequences are those reported by Narita and Ishii (1962) and Niu and Fraenkel-Conrat (1955) respectively. We have confirmed the report of Satake, Sasakawa, and Honda (1965) that both Pronase and Nagarse catalyse a similar limited digestion, and a large plakalbumin peptide of the same amino acid composition as discussed in this paper is formed.

Preliminary experiments using trypsin and chymotrypsin did not fragment the plakalbumin peptide to soluble peptides which could be readily fractionated. The action of Nagarse and pepsin on the separated peptide resulted in more fragments, and digests obtained with these enzymes are being investigated.

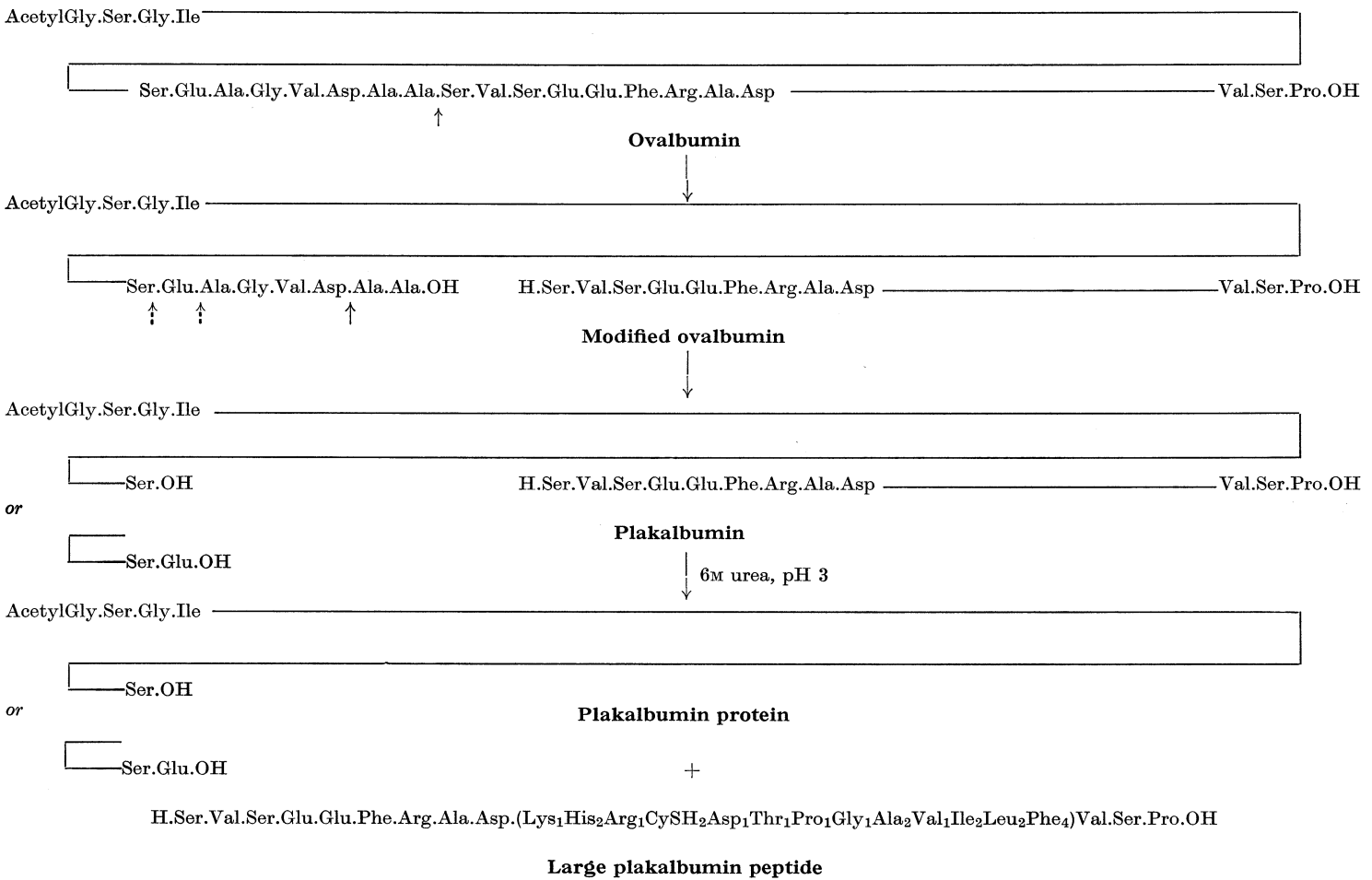

Fig. 1.—Probable course of limited proteolysis of ovalbumin by subtilopeptidase A. ↑ Major site of enzyme attack. ⇡ Other bonds split by enzyme.

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