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SEPARATION OF THE TRYPTIC PEPTIDES FROM REDUCED, ALKYLATED HEN EGG WHITE LYSOZYME BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We describe high-performance liquid chromatographic systems for the separation of the tryptic peptides of reduced, alkylated hen eggwhite lysozyme. The resolved peptides which contained 3–23 amino acid residues were identified by determination of amino acid composition. Gradients of acetonitrile with aqueous ammonium acetate or ammonium chloride were employed to elute from a reversed-phase C_{18} column, monitoring absorbance at 205 nm, 212 nm or 280 nm. Peptides containing S-carboxymethyl-cysteine eluted more rapidly than the corresponding S-ethylsuccinimido peptides.

INTRODUCTION

As part of a series of investigations to determine the folding pathway of reduced lysozyme, an analytical method for tryptic peptides of that protein was required. The proposed application involves separating, estimating, and identifying cysteine-containing peptides in the series of intermediates that arise in the oxidation of the reduced protein. We wanted a method that was rapid, reliable, and sensitive, with high resolving power. For further examination of peptide fractions, it is also desirable to desalt the fractions by sublimation.

At the time these studies were begun, relatively few high-performance liquid chromatographic (HPLC) separations of tryptic peptide mixtures had been achieved[1–3,4]. After unsuccessfully attempting to adapt various published systems to our objective, we began to get encouraging results with a reversed-phase C_{18} column packing and gradient elution with ammonium salt and acetonitrile. Development of the systems was assisted by pilot reversed-phase thin-layer chromatographic (TLC) experiments. Hen egg white lysozyme (HEWL) has 129 amino acids and should theoretically yield 14 tryptic peptides as well as free lysine, arginine and leucine. In this paper we describe HPLC gradient systems which resolve most of the tryptic peptides of reduced alkylated lysozyme. The identities of the peptides have been established by amino acid analysis. While several recent publications have demonstrated the ability of reversed-phase systems to separate tryptic peptides[1,3–8] only a

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few studies have determined the amino acid composition of tryptic peptides collected from HPLC[2,3,8–13]. We believe that our experience in developing successful systems will be useful to other investigators.

MATERIALS AND METHODS

General methods

Sephadex G-25 medium and SP-Sephadex C-25-120 were obtained from Pharmacia (Uppsala, Sweden). Amberlite AG 1-X2, 200–400 mesh, was from Bio Rad Labs. (Richmond, CA, U.S.A.).

Effluents from Sephadex and ion-exchange columns were monitored for absorbance at 230 nm on a Cecil Model CE 272 ultraviolet spectrophotometer (Cecil, Cambridge, Great Britain). Subsequently the fractions were read manually at 280 nm on a Zeiss Model MAQ III spectrophotometer (Oberkochen, Württenberg, G.F.R.).

Column fractions were collected on an LKB 7000 Ultrarac fraction collector (LKB, Stockholm, Sweden).

For amino acid analysis degassed samples were hydrolyzed at 110°C with either 6 *M* hydrochloric acid or 4 *M* methanesulfonic acid (Pierce, Rockford, IL, U.S.A.) for 24–72 h. A Beckman Model 119C amino acid analyzer (Beckman, Palo Alto, CA, U.S.A.) equipped with a single column of Beckman Type AA-20 resin was employed.

HPLC separations were performed on a Varian Model 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a Vari-chrom variable-wavelength detector and a Fischer Recordall Series 5000 dual-pen recorder (Fisher Scientific, Pittsburgh, PA, U.S.A.). A Varian reversed-phase Micropak MCH-10 column (30 cm × 4 mm I.D.) was employed with a guard column of Vydac RP resin. The reagents were analytical grade and the ammonium salt buffers were prefiltered through a Millipore Type HA 0.45-μm filter. Water was purified on a Milli-Q system containing the following cartridges: one activated carbon, two mixed-bed deionization, and one 0.22-μm membrane filter (Millipore, Bedford, MA, U.S.A.). HPLC-grade acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of tryptic digests of reduced, S-alkylated HEWL

The four disulfides of HEWL (Miles Labs., South Africa) were reduced with dithiothreitol at pH 8.6 in the presence of urea and EDTA as previously described[14]. The reduced lysozyme was S-alkylated with either N-ethylmaleimide[15] or iodoacetic acid[16] to form respectively S-ethylsuccinimido lysozyme (ES_8LZM) and S-carboxymethyl lysozyme (CM_8LZM). Complete S-alkylation was confirmed by amino acid analysis and by a negative reaction with 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent).

Tryptic digestion of the S-alkylated lysozyme preparations was performed essentially by the method of Canfield[17]. Samples of 10–100 mg were dissolved at final concentrations of 0.05–1.0% in 0.10 *M* acetic acid, 10^{-3} *M* in calcium chloride. The pH was adjusted to 7 with dilute ammonium hydroxide. Diphenylcarbamyl chloride (DPCC)-treated trypsin (Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.10 *M* acetic acid to a concentration of 1 mg/ml. Two separate additions of trypsin were

made to total 2–3% of the weight of the substrate. The pH was maintained and the digestion carried out for 18–24 h. The digestion was stopped by lyophilization.

Preliminary fractionation of the CM_8LZM and ES_8LZM tryptic peptides on a Sephadex G-25 medium column

The lyophilized tryptic digests from 10–24 mg of CM_8LZM or ES_8LZM were dissolved in approximately 3 ml of 0.10 *M* acetic acid and applied to a Sephadex G-25 medium column (110 × 2.5 cm I.D.). The peptides were eluted with 0.10 *M* acetic acid. Fractions (2 min) containing 5–6 ml were collected. Fractions were pooled, lyophilized and reconstituted in 3–6 ml of 0.10 *M* acetic acid for HPLC chromatography.

Preliminary anion-exchange chromatography of the CM_8LZM and ES_8LZM tryptic peptides

The lyophilized tryptic digests from 50 mg of CM_8LZM or ES_8LZM were dissolved in 5–10 ml of 0.10 *M* acetic acid and applied to a SP-Sephadex (NH_4^+) column (46 × 1.5 cm). The peptides were eluted first with a three-chamber gradient of 500 ml each 0.040 *M* ammonium acetate (pH 3.80), 0.060 *M* ammonium acetate (pH 4.10) and 0.080 *M* ammonium acetate (pH 4.50) and then with a two-chamber gradient of 500 ml each 0.080 *M* ammonium acetate (pH 4.50) and 0.15 *M* ammonium acetate (pH 5.50). The column was washed finally with a 0.15 *M* ammonium acetate buffer made 4 *M* in urea and 0.10 *M* in sodium chloride. Fractions (7 min) of approximately 8.5 ml were collected. UV-Absorbing fractions (10 μl) were examined by HPLC. Those giving no peak on HPLC or a single HPLC peak were hydrolyzed (1 ml) and analyzed for amino acid composition. HPLC chromatography itself was used to resolve multipeak fractions.

Preliminary cation-exchange chromatography of the CM_8LZM and ES_8LZM tryptic peptides

The lyophilized tryptic peptides from 50 mg CM_8LZM or ES_8LZM were dissolved in 3–5 ml of 0.01 *M* ammonium acetate and applied to an Amberlite AG 1-X2 (CH_3COO^-) column (46 × 1.5 cm I.D.). The peptides were eluted with a three-chamber gradient of 500 ml each; 0.050 *M* acetic acid, 0.50 *M* acetic acid and 2.00 *M* acetic acid. The column was washed finally with an additional 500 ml of 2 *M* acetic acid. Fractions (3 min) of approximately 4.2 ml were collected. UV-Absorbing fractions (10 μl) were examined directly by HPLC and those showing no peak or a single 205 nm absorbance peak were hydrolyzed (1 ml) and analyzed for amino acid composition. Multipeak fractions were resolved by HPLC chromatography.

HPLC gradient systems for mapping tryptic peptides

Separation of the tryptic peptides on HPLC was first achieved using either 0.10 *M* ammonium acetate or 0.10 *M* ammonium chloride, pH 4.1 (reservoir A) and acetonitrile (reservoir B) in the following gradient program: 0–10 min, 5–22% B; 10–12 min, 22–24% B; 12–14 min, 24% B; 14–19 min, 24–28% B; 19–25 min, 28–36% B (system I). The conditions were: flow-rate, 1.5 ml/min; chart speed, 1 cm/min; UV detection, 280 nm for the ammonium acetate system and 205 nm for the ammonium chloride system; range, 0.05 a.u.f.s. for ammonium acetate and 0.10 units for ammonium chloride.

A modified system used 0.010 *M* ammonium chloride or 0.010 *M* ammonium acetate, pH 4.2 (reservoir A) and acetonitrile (reservoir B) in the following program: 0–20 min, 5–25% B; 20–30 min, 25–40% B (system II). Other conditions were the same as for system I, except that for ammonium acetate 212 nm detection could be used with a full scale range of 0.2 absorbance unit.

When HPLC fractions were collected, ammonium acetate buffers were used. For System I, peptides which did not absorb at 280 nm were collected by time based on their elution profile in the chloride system. For collection the back-pressure restrictor and exit line from the Vari-chrom detector were replaced with tubing having a total volume of 400 μl. Injections of the different peptide preparations were made and the peaks collected manually. For the Sephadex G-25 column pool samples, peaks from six to ten 10 μl injections were combined, lyophilized, hydrolyzed and the entire sample applied to the amino acid analyzer (0–5 nmol scale). For fractions from the ion-exchange columns, peaks from a single injection of 2–10 μl were collected for amino acid analysis.

RESULTS AND DISCUSSION

The preliminary fractionation of a tryptic digest of ES_8LZM using Sephadex G-25 is shown in Fig. 1. The fractions were pooled as shown, following Anderson and Wetlaufer[18]. Tryptic digests of CM_8LZM gave similar elution profiles to those of ES_8LZM on Sephadex G-25. Pool D had an elution volume equal to the total column volume, suggesting a hydrophobic composition. Both pools C and D have higher 280 nm absorbance than pools A and B.

The HPLC gradient system using acetonitrile and 0.10 *M* ammonium chloride was developed to resolve the peptides in these four pools. Fig. 2 shows the elution profiles of these pools and Tables I and II give the amino acid analyses of the collected

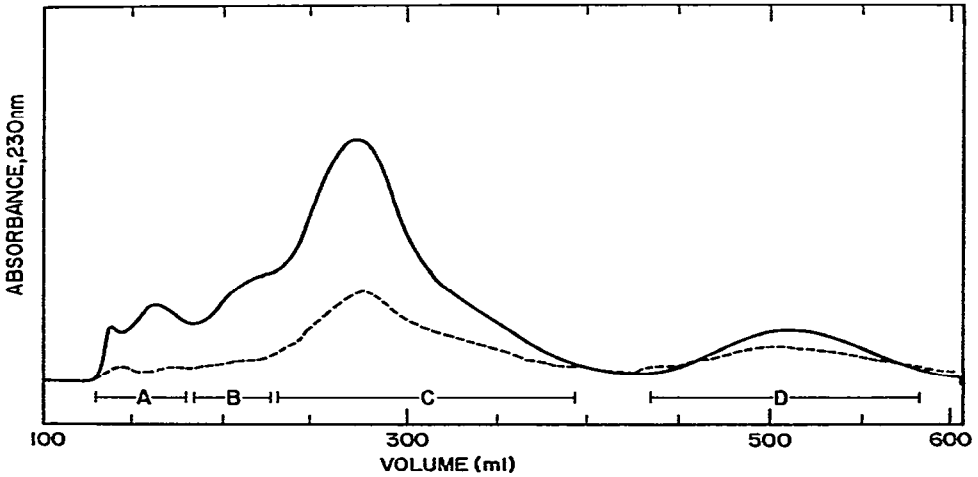

Fig. 1. The preliminary separation of the tryptic peptides from S-ethylsuccinimido lysozyme by Sephadex G-25 chromatography. The column was eluted with 0.10 *M* acetic acid and four peptide pools A, B, C and D were made by combining fractions as indicated. ——, Absorbance at 230 nm; – – –, absorbance at 280 nm.

peaks. Peptide assignments were made considering the known sequence of HEWL[17]. The three major peaks from pool B of the ES_8LZM digest (Fig. 2A) were identified as peptides Phe^{34}–Arg^{45} and a doublet of ES-Cys^{6}–Lys^{13} and Asn^{46}–Arg^{61}. The following peptides were found in pool C: Thr^{69}–Arg^{73}, His^{15}–Arg^{21}, Gly^{117}–Arg^{125}, Ile^{98}–Arg^{112} and Gly^{22}–Lys^{33}. The latter two peptides elute as one peak with ammonium chloride, but separate well with ammonium acetate (Fig. 2B). Pool D contained essentially pure Trp^{62}–Arg^{68} (Fig. 2C). The order of elution of the tryptic peptides from the Sephadex G-25 column is consistent with conventional expectations. The pool B peptides have eight, twelve and sixteen amino acid residues, and contain no tryptophan. The three large peptides found in pool C have nine, twelve, and fifteen amino acid residues, including one or two tryptophans. Trp^{62}–Arg^{68} has seven amino acids, two of which are tryptophan, making plausible its retention on Sephadex G-25.

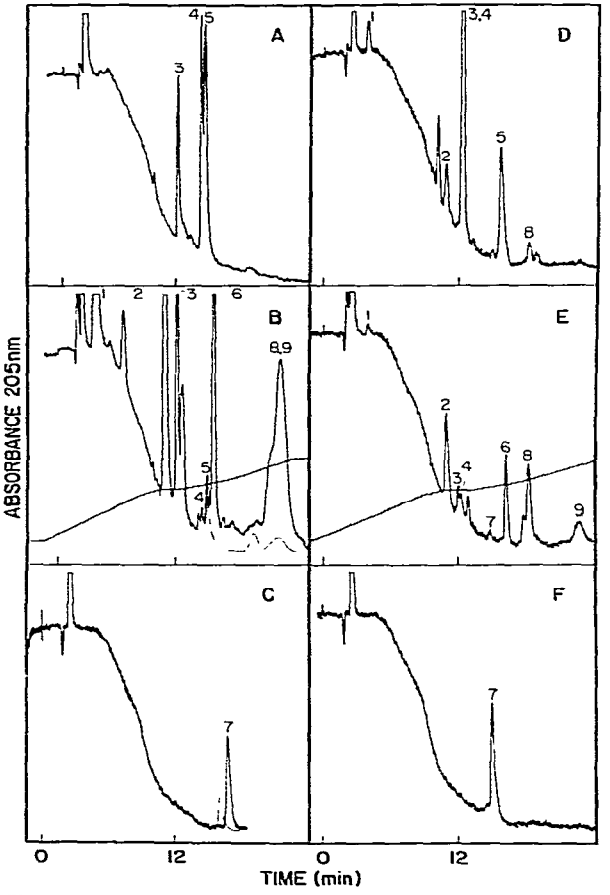

Fig. 2. HPLC separation of the lysozyme tryptic peptide pools collected from Sephadex G-25 medium chromatography. HPLC gradient system I with ammonium chloride was used as described in the text. (A), Pool B from ES_8LZM digest; (B), pool C from ES_8LZM digest; (C), pool D from ES_8LZM digest; (D), pool B from CM_8LZM digest; (E), pool C from CM_8LZM digest and (F), pool D from CM_8LZM digest. The numbers above the peaks refer to the peptides identified in the tables. The gradient pen is offset 1.5 min to the left of the actual elution time. The dashed curves show the tryptophan peptides with ammonium acetate in the eluent.

TABLE I

THE AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF N-ETHYLMALEIMIDE BLOCKED HEN EGG WHITE LYSOZYME SEPARATED BY HPLC

The data are given in residues/mole. The numbers in parentheses refer to the number of residues expected from the known sequence of lysozyme. Residue numbers are given after hydrolysis in 4 *M* methane sulfonic acid.

Amino acids	*HPLC peak No. (Fig. 2)*								
	1	*2*	*3*	*4 and 5*	*6*	*7*	*8*	*9*	*Between 2 and 3 10*
	Peptide(s) identified								
	Thr^{69}–Arg^{73}	*His^{15}–Arg^{21}*	*Phe^{34}–Arg^{45}*	*Cys^{6}–Lys^{13} and Asn^{46}–Arg^{61}*	*Gly^{117}–Arg^{125}*	*Trp^{62}–Arg^{68}*	*Gly^{22}–Lys^{33}*	*$Ileu^{98}$–Arg^{112}*	*Lys^{1}–Arg^{5}*
S-Ethyl-succinimido cysteine				0.4 (1)		1.4 (1)	0.6 (1)	1.6	
Aspartic acid		2.2 (2)	3.3 (3)	3.6 (4)	1.3 (1)	2.3 (2)	1.3 (1)	3.2 (3)	0.2
Threonine	0.9 (1)		1.9 (2)	2.1 (2)	1.1 (1)				
Serine	0.9 (1)	0.2	1.1 (1)	2.0 (2)			0.7 (1)	1.3 (1)	
Glutamic acid			2.5 (2)	2.4 (2)	1.2 (1)		0.1	0.4	0.3
Proline	1.0 (1)								
Glycine	1.2 (1)	1.3 (1)	1.0	2.5 (2)	1.4 (1)	1.3 (1)	2.7 (2)	2.8 (2)	0.7 (1)
Alanine			1.9 (1)	2.8 (3)	1.2 (1)		2.5 (2)	2.3 (2)	
Valine					0.8 (1)		1.5 (1)	2.0 (2)	0.5 (1)
Methionine				0.8 (1)				0.4 (1)	
Isoleucine				1.7 (2)	0.9 (1)		0.4	0.6 (1)	
Leucine		1.1 (1)		2.1 (2)			1.1 (1)		0.2
Tyrosine		0.7 (1)		0.8 (1)			0.7 (1)		0.4
Phenylalanine			2.5 (2)						0.7 (1)
Histidine		1.0 (1)	0.2						
Lysine			0.2	0.9 (1)			1.0 (1)		0.6 (1)
Arginine	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.2	1.0 (1)	1.0 (1)
*Tryptophan					0.9 (1)	1.6 (2)	+ (1)	+ (2)	

* Tryptophan is indicated present after 6 *M* HCl hydrolysis by +.

TABLE II

THE AMINO ACID COMPOSITION OF TRYPTIC PEPTIDES OF IODOACETIC ACID BLOCKED HEN EGG WHITE LYSOZYME SEPARATED BY HPLC

The data are given in residues/mole. The numbers in parentheses refer to the number of residues expected from the known sequence of lysozyme.

Amino acids	*HPLC peak No. (Fig. 2)*							
	1	*2*	*3 and 4*	*7*	*5*	*6*	*8*	*9*
	Peptide(s) identified							
	Thr^{69}–Arg^{73}	*His^{15}–Arg^{21}*	*Cys^{6}–Lys^{13} and Phe^{34}–Arg^{45}*	*Try^{62}–Arg^{68}*	*Asn^{46}–Arg^{64}*	*Gly^{117}–Arg^{125}*	*Gly^{22}–Lys^{33}*	*$Ileu^{98}$–Arg^{112}*
Carboxymethylcysteine			0.8 (1)	1.3 (1)			1.1 (1)	
Aspartic		2.0 (2)	2.5 (3)	2.4 (2)	4.0 (4)	1.5 (1)	2.1 (1)	2.6 (3)
Threonine	1.1 (1)		1.9 (2)		2.0 (2)	1.2 (1)		
Serine	1.1 (1)	0.8	1.0 (1)	0.1	2.0 (2)	1.4	1.5 (1)	1.1 (1)
Glutamic acid		0.8	2.8 (3)		1.6 (1)	1.6 (1)		0.6
Proline	1.1 (1)						0.3	
Glycine	1.2 (1)	2.1 (1)	0.6	1.0 (1)	2.7 (2)	2.4 (1)	2.5 (2)	2.6 (2)
Alanine		0.5	4.5 (4)		0.9	1.5 (1)	2.1 (2)	2.0 (2)
Valine						1.1 (1)	1.3 (1)	1.9 (2)
Methionine			0.9 (1)					1.5 (1)
Isoleucine					3.0 (2)	1.0 (1)		1.7 (1)
Leucine		1.2 (1)	1.6 (1)		2.2 (1)	0.2	1.1 (1)	
Tyrosine		1.0 (1)			1.9 (1)		1.0 (1)	
Phenylalanine			1.4 (2)					
Histidine		0.9 (1)						
Lysine	0.1		1.2 (1)				1.0 (1)	
Arginine	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)		0.7 (1)
Tryptophan				1.5 (2)		0.7 (1)	1.1 (1)	1.3 (2)

All the tryptophan-containing peptides elute late in the HPLC gradient, consistent with their relatively higher hydrophobicity. In some chromatograms of the pool C peptides a peak is seen between His^{15}–Arg^{21} and Phe^{34}-Arg^{45}; this has been identified as Lys^{1}–Arg^{5}, showing that trypsin did not completely cleave the Lys^{1}–Val^{2} bond. This peptide has been found previously in tryptic digests of lysozymes[17,19].

For comparison, the HPLC maps of the corresponding pools from a CM_8LZM digest are shown in Fig. 2D–F. Pool B contains Asn^{46}–Arg^{61} and a single peak of both Phe^{34}–Arg^{45} and CM-Cys^{6}–Lys^{13}. Pool C had the same peptide content as the corresponding ES_8LZM pool; likewise pool D was Trp^{62}–Arg^{68} (Table II). The three S-carboxymethylated cysteinyl peptides (Cys^{6}–Lys^{13}, Gly^{22}–Lys^{33} and Try^{62}–Arg^{68}) elute more quickly than the corresponding S-ethylsuccinimido cysteinyl peptides. The amino acid analyses of the pool B peptides (Tables I and II) show the CM-Cys^{6}–Lys^{13} peptide eluting with Phe^{34}–Arg^{45}, the ES-Cys^{6}–Lys^{13} peptide elutes later, very close to Asn^{46}–Arg^{61}. The second half of this doublet (peaks 4 and 5) elutes in

TABLE III

THE AMINO ACID COMPOSITION OF OTHER TRYPTIC PEPTIDES OF HEN EGG WHITE LYSOZYME SEPARATED BY ION-EXCHANGE CHROMATOGRAPHY

The data are given in residues/mole. The numbers in parentheses refer to the number of residues expected from the known sequence of lysozyme.

Amino acids	*HPLC peak No. (Figs. 2 and 3)*					
	11	*11*	*12*	*13*	*10*	*3*
	Peptide identified					
	Gly^{126}–Arg^{128} (CM)	*Gly^{126}–Arg^{128} (ES)*	*Gly^{126}–Leu^{129} (CM)*	*Val^{2}–Arg^{5}*	*Lys^{1}–Arg^{5}*	*Phe^{34}–Arg^{45}*
S-Carboxymethyl-cysteine	0.9 (1)		0.7 (1)			
S-Ethylsuccinimido-cysteine		0.6 (1)				
Aspartic acid	0.2	0.2				2.7 (3)
Threonne						1.9 (2)
Serine						1.3 (1)
Glutamic acid	0.1					2.0 (2)
Proline						
Glycine	1.0 (1)	1.2 (1)	1.2 (1)	1.2 (1)	0.9 (1)	0.5
Alanine		0.2	0.3	0.4	0.2	1.4 (1)
Valine				0.7 (1)	1.3 (1)	
Methionine						
Isoleucine						
Leucine	0.1	0.2	1.1 (1)		1.0 (1)	
Tyrosine						
Phenylalanine				0.7 (1)	0.8 (1)	1.6 (2)
Histidine						
Lysine		0.2				
Arginine	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)
Tryptophan						

the same position as Asn^{46}–Arg^{61} in the carboxymethylated pool, allowing the identity and positions of the three peptides to be deduced. In pool C the carboxymethylated form of Gly^{22}–Lys^{33} is readily separated from Ile^{98}–Arg^{112} in either chloride or acetate buffer, unlike the S-ethylsuccinimido peptide (Fig. 2B and E). The S-ethylsuccinimido derivative of Trp^{62}–Arg^{68} is well separated from the other peptides while the carboxymethylated form elutes close to Asn^{46}–Arg^{61} and Gly^{117}–Arg^{125} (Fig. 2C–F). Fortunately, this overlap can be avoided by preliminary fractionation on Sephadex G-25.Therefore, by suitable choice of alkylating agent, improved separation and isolation of peptides is achieved. The longer retention times of the ES peptides suggests that the S-ethylsuccinimido group is less polar than the carboxymethyl group.

Preliminary fractionation of HEWL tryptic peptides by ion-exchange chromatography provided independent confirmation of the peak assignments made in Tables I and II. The identification of the doublet peaks 4 and 5 of ES_8LZM digest pool B (Fig. 2A) as ES-Cys^6–Lys^{13} and Asn^{46}–Arg^{61} (Table I) was confirmed by the injec-

4	*14*	*14*	*15*	*15*	*16*	*17*
CM-Cys^6–Lys^{13}	*CM-Cys^6–Arg^{14}*	*ES-Cys^6–Arg^{14}*	*Asn^{74}–Lys^{96} (CM_3)*	*Asn^{74}–Lys^{96} (ES_3)*	*Val^{109}–Arg^{112}*	*Gly^{117}–Trp^{123}*
1.1 (1)	0.9 (1)		3.1 (3)			
		0.7 (1)		1.5 (3)		
	0.3	0.3	3.6 (4)	4.2 (4)	0.2	1.3 (1)
		0.1	1.0 (1)	1.3 (1)		0.9 (1)
		0.2	3.9 (4)	4.4 (4)		0.4
1.1 (1)	1.2 (1)	1.2 (1)				0.9 (1)
			1.0 (1)	1.2 (1)		
	0.3	0.4	0.4	0.4	0.2	1.3 (1)
3.0 (3)	3.4 (3)	2.7 (3)	3.1 (3)	3.4 (3)	1.1 (1)	1.4 (1)
			0.9 (1)	1.3 (1)	0.8 (1)	1.0 (1)
1.0 (1)	0.8 (1)	1.0 (1)				
			1.9 (2)	1.8 (2)		
1.1 (1)	0.7 (1)	1.1 (1)	2.8 (3)	2.6 (3)		
0.5						
1.0 (1)	1.0 (1)	0.8 (1)	1.0 (1)	1.0 (1)		
	1.0 (1)	0.9 (1)			1.0 (1)	
					0.9 (1)	1.0 (1)

tions of SP-Sephadex pool 139–154 (ES-Cys^{6}–Lys^{13}) and pool 42–46 (Asn^{46}–Arg^{61}) which gave peaks 4 and 5 respectively. Similarly, the identification of a peak of CM_8LZM digest pool B (Fig. 2D) as containing both CM-Cys^{6}–Lys^{13} and Phe^{34}–Arg^{45} was verified by both amino acid analyses of Amberlite AG 1-X2 fractions 21–40 and 99–106 (Table III) and co-chromatography in HPLC System I.

The preliminary fractionation of HEWL tryptic peptides by ion-exchange chromatography also provided additional HPLC peak assignments (Table III). Some of these are peptides resulting from incomplete trypsin cleavage where there are adjacent cleavage sites. These peptides have been reported previously in the literature[17,19,20]. Two peptides Gly^{117}–Trp^{123} and Val^{109}–Arg^{112} require cleavages of Trp–Val and Ile–Trp, bonds not expected to be cleaved by DPCC-treated trypsin.

The elution profiles found for HEWL tryptic peptides from the ion-exchange columns were similar to those found for cation-exchange chromatography by Canfield[17] and Jolles *et al.*[19] and for anion-exchange chromatography by Anderson and Wetlaufer[18] and Fujio *et al.*[21]. The omission of pyridine from the buffer systems somewhat altered the order of elution but did not appreciably affect the yields of peptides. The absence of pyridine did allow the direct monitoring of peptide elution by absorbance at 230 nm; peptides of three or more residues were easily detected.

While HPLC System I has been useful in separating the peptides containing 5–16 amino acid residues, it suffers two limitations. First, the peptide Asn^{74}–Lys^{96}, known to be in pool A, is not eluted. Although it contains three cysteinyl residues neither alkylated form could be consistently eluted. Second, the free amino acids and smaller peptides were not separated. An experiment was performed using the material eluting early from the HPLC run of CM-pool C to demonstrate their presence as a group. Sequentially timed collections were made between 1.5 and 3.5 min and amino acid analyses on each obtained. In this way the presence of Cys^{115}–Lys^{116}, free arginine, free lysine, free leucine, Asp^{113}–Arg^{114}, Gly^{126}–Cys^{127}–Arg^{128} and Val^{2}–Phe^{3}–Gly^{4}–Arg^{5} was demonstrated. Thus the remainder of the set of theoretical tryptic peptides from HEWL were accounted for.

The Amberlite AG 1-X2 chromatography gave better resolution for the CM_8LZM digest and the SP-Sephadex chromatography was more effective for the ES_8LZM digest. CM-cysteine is more acidic than ES-cysteine; therefore, the CM_8LZM digest has more neutral and acidic peptides while the ES_8LZM digest has more basic peptides. Peptides separated by ion exchange are often examined and further purified by paper chromatography or TLC and/or electrophoresis prior to amino acid analysis. HPLC chromatography proved to be an excellent alternative for this purpose.

HPLC Gradient system II proved to give an excellent overall resolution of the tryptic peptides of reduced, alkylated HEWL. The results of injection of unfractionated tryptic digests are shown in Fig. 3. The identification of the peaks (see Tables I, II and III) was deduced by injections of Sephadex G-25 pools and ion-exchange column fractions, and by comparison of the absorbances at 280 nm and 205 nm to further verify the tryptophan-containing peptides. The Phe^{34}–Arg^{45} and CM-Cys^{6}–Lys^{13} peaks, which eluted together in system I, were well-resolved in system II. Peptides Trp^{62}–Arg^{68} (CM), Asn^{46}–Arg^{61}, and Gly^{117}–Arg^{125} which clustered in the first system were now well separated. Ile^{98}–Arg^{112} and Gly^{22}–Arg^{33} (ES) separate in either chloride or acetate, using system II. The largest lysozyme peptide Asn^{74}–Lys^{96},

blocked with either alkylating agent, did elute in system II, although the tricarboxymethylated derivative coelutes with Gly^{22}–Lys^{33} (CM). The two tetrapeptides (Val^{2}–Arg^{5} and Gly^{126}–Leu^{129}) and the tripeptide (Gly^{126}–Arg^{128}) were resolved and identified by amino acid analysis (Table III). Work to locate and identify the dipeptides is still in process. Collection and identification of these small peptides should proceed easily since direct detection at 212 nm is possible with the low acetate concentration of system II.

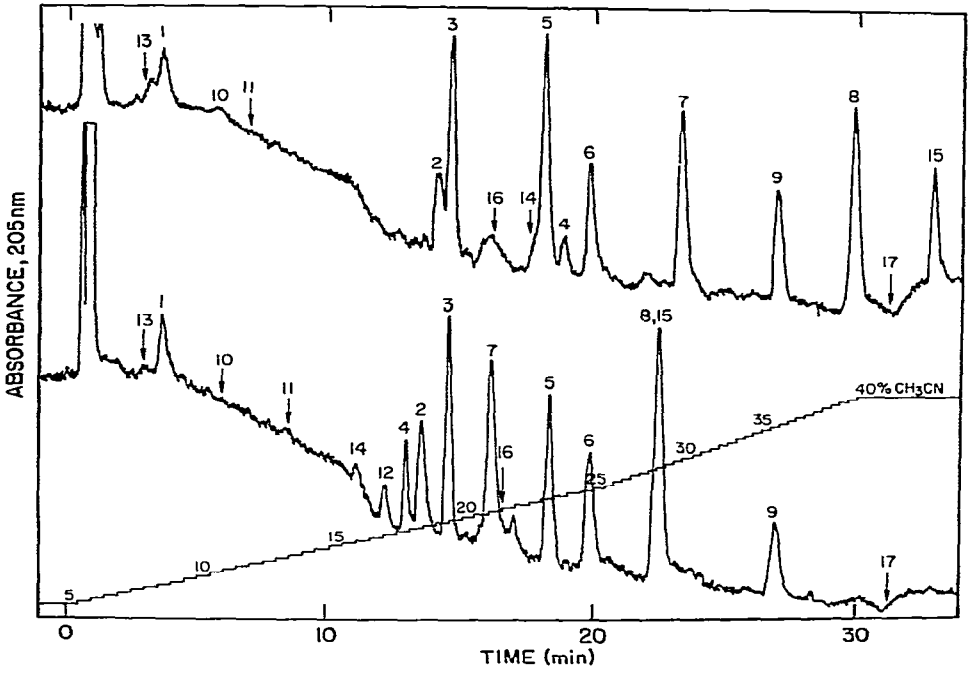

Fig. 3. HPLC separation of the unfractioned tryptic peptides of HEWL, using HPLC gradient system II with ammonium chloride as described in the text. The top tracing is the separation of ES_8LZM digest and the bottom that of CM_8LZM digest. In each case a digest of 1.3 μg or 90 pmol lysozyme was injected. The numbers above the peaks refer to the peptides identified in the tables. The gradient pen is 1.5 min to the right of the actual elution time.

A general comment as to the stability of the Varian MCH-10 column, packed with uncapped C_{18} material, seems warranted. Our column has been in constant use for 2 years. It has been used with other salts and with methanol as an alternative organic solvent. The column is stored in 100% acetonitrile after thoroughly washing the salt out of the system. The Sephadex column pool C peptides have been used as an internal standard. Their elution pattern has been constant once the column has been re-equilibrated. We have found that as much as four hours is necessary for aqueous acetonitrile equilibration after methanol use. The guard column resin is changed every 6 to 8 weeks.

The purposes of this investigation were twofold. First to develop a system(s) suitable for the separation of a group of tryptic peptides from lysozyme which might be useful for other protein digests. Second to modify cysteine residues within these

peptides with two different kinds of alkylating agents to determine which derivative was more suitable for resolution on a reversed-phase column. The use of ammonium salts and an acetonitrile gradient has resulted in the separation of a large number of peptides containing 3–23 amino acid residues. While our system was being developed Coy[9] published a figure showing the separation of the tryptic peptides of β_h-endorphin on LiChrosorb RP-18 with a linear gradient of 10–50% isopropanol–ammonium acetate, pH 4. The author states that substitution of acetonitrile for isopropanol improved resolution. This system, which is similar to ours, was used to separate only five peptides, while in the present study as many as seventeen peptides are separated. Two HPLC separations on μBondapak C_{18} of tryptic peptides from hemoglobin variants[11,12] have recently been published. They also employ ammonium acetate (pH 6.07 and pH 5.7) and acetonitrile gradients. Our substitution of ammonium chloride for ammonium acetate in order to detect peaks at 205 nm showed the two salts to be comparable eluants. We conclude therefore that ammonium salt (pH 4–6 range)–acetonitrile systems are generally effective for peptide mapping. The sensitivity found here for peptide detection by 205 nm absorbance appears to be about the same as that reported by Rubinstein and co-workers[2,8] employing fluorescence detection. Our studies also show that S-ethylsuccinimido- and S-carboxymethyl-cysteinyl peptides are equally well resolved on reversed-phase columns but their different retention times can be useful in improving the overall separation of a group of peptides. It seems likely that our systems will be applicable to other peptide mixtures.

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REFERENCES

1 I. Molnar and Cs. Horváth, *Pept. Proc. Amer. Pep Symp., 5th*, (1977) 48.
2 M. Rubinstein, S. Stein and S. Udenfriend, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 4969.
3 C. Schwabe and J. K. McDonald, *Biochem. Biophys. Res. Commun.*, 74 (1977) 1501.
4 W. S. Hancock, C. A. Bishop, R. L. Prestidge and M. T. W. Hearn, *Anal. Biochem.*, 89 (1978) 203.
5 M. T. W. Hearn and W. S. Hancock, *Trends Biochem. Sci.*, 4 (1979) N58.
6 W. L. Hollaway, A. S. Bhown, J. E. Mole and J. C. Bennett, in G. L. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography*, Marcel Dekker, New York, Basel, 1979, p. 163.
7 G. J. Hughes, K. H. Winterhalter and K. J. Wilson, *FEBS Letters*, 108 (1979) 81.
8 M. Rubinstein, S. Cheng-Kiang, S. Stein and S. Udenfriend, *Anal. Biochem.*, 95 (1979) 117.
9 D. H. Coy, in G. L. Hawk (Editor), *Biological/Biomedical Applications of Liquid Chromatography, II*, Marcel Dekker, New York, Basel, 1979, p. 283.
10 C. S. Fullmer and R. H. Wasserman, *J. Biol. Chem.*, 254 (1979) 7208.
11 W. A. Schroeder, J. B. Shelton, J. R. Shelton and D. Powars, *J. Chromatogr.*, 174 (1979) 385.
12 J. B. Wilson, H. Lam, P. Pravatmuang and T. H. J. Huisman, *J. Chromatogr.*, 179 (1979) 271.
13 I. M. Chaiken and C. J. Hough, *Anal. Biochem.*, 107 (1980) 11.
14 S. S. Ristow and D. B. Wetlaufer, *Biochem. Biophys. Res. Commun.*, 50 (1973) 544.
15 E. R. Johnson, W. L. Anderson, D. B. Wetlaufer, C. Lee and M. Z. Atassi, *J. Biol. Chem.*, 253 (1978) 3408.
16 T. E. Creighton, *J. Mol. Biol.*, 129 (1979) 411.
17 R. E. Canfield, *J. Biol. Chem.*, 238 (1963) 2691.
18 W. L. Anderson and D. B. Wetlaufer, *J. Biol. Chem.*, 251 (1976) 3147.
19 J. Jolles, E. VanLeemputten, A. Mouton and P. Jolles, *Biochim. Biophys. Acta*, 257(1972) 497.
20 T. V. Chentsova and N. A. Kravchenko, *Biokhimiya*, 43 (1978) 1977.
21 H. Fujio, R. E. Martin, Y. Ha, N. Sakato and T. Amano, *Biken J.*, 17 (1974) 73.