Effect of Sequential Degradation on the Haptenic Activity of a Tryptic Peptide Isolated from Reduced and Alkylated Egg-White Lysozyme and the Haptenic Properties of Its Amino-Terminal Residues*

Karen E. Thompson† and Julia Gerwing Levy

ABSTRACT: The tryptic peptide T-11 isolated from CM-lysozyme was shown previously to have haptenic activity in the CM-lysozyme immune system. Sequential degradation of this peptide from the NH_2 terminus by Edman degradation or by leucine amino peptidase digestion demonstrated that removal of amino acids from this part of the molecule impaired its haptenic behavior. Removal of the two COOH-terminal amino acid residues with carboxypeptidases A and B had no effect on the immunological activity of this peptide.

The synthesis of the NH_2-terminal decapeptide of T-11 and subsequent demonstration of its haptenic properties confirmed the participation of this region of the T-11 peptide in its immunological activity.

Studies on the antigenic properties of egg-white lysozyme, both native and alkylated, have been quite extensive over the past 2 years. Two regions have been of immunologic interest in both these forms of the molecule. In the native molecule, Fujio *et al.* (1968) and Shinka *et al.* (1967), respectively, demonstrated two immunologically active peptic peptides, one containing both the NH_2- and COOH-terminal peptides Lys^{1}–Asn^{27} and Ala^{122}–Leu^{129} linked by a disulfide bond at positions 6 and 127, and the other containing the residues Gln^{57}–Ala^{107}. Arnon and Sela (1969) prepared a specific immunoadsorbent by conjugating the loop peptide of native lysozyme, constituting the sequence Cys^{64}–Leu^{83} joined by a disulfide bond between residues 64 and 80 with bromoacetyl-cellulose, and isolated antibodies directed against the loop peptide from antiserum to native lysozyme, implicating this region as an antigenic determinant in the native molecule.

Parallel work has been done on reduced and carboxymethylated lysozyme (CM-lysozyme), which does not cross-react immunologically to an appreciable degree with the native lysozyme system (Gerwing and Thompson, 1968). Young and Leung (1969) showed that cyanogen bromide cleavage of CM-lysozyme yielded two immunologically active peptides containing both the NH_2- and COOH-terminal peptides comprising residues 1–12 and 106–129 which overlapped the regions found to be antigenic in native lysozyme. These peptides, while inhibiting complement fixation between CM-lysozyme and its homologous antiserum, were not able to inhibit the reaction between native lysozyme and its antiserum. In this laboratory, we demonstrated haptenic activity of a tryptic peptide (T-11) of CM-lysozyme encompassing the sequence Asn^{74}–Lys^{96} (Gerwing and Thompson, 1968), which also was unable to inhibit complement fixation between native lysozyme and its homologous antiserum. It may be fortuitous, but is interesting to note that this peptide also falls within the second region found to be antigenic in native molecule.

The present paper includes further research on the peptide T-11 in which the participation of the NH_2- and COOH-terminal region in its haptenic behavior has been studied.

Materials and Methods

Preparation of T-11. CM-lysozyme and its tryptic peptides were prepared according to the method of Canfield and Anfinsen (1963) and Canfield (1963). The modified method for the purification of the T-11 peptide has been described previously (Gerwing and Thompson, 1968).

Preparation of Antiserum and Immunological Assays. Rabbit antiserum was prepared and pooled according to previously described methods (Gerwing and Thompson, 1968). Hapten inhibition measurements in this study involved the ability of various preparations to inhibit precipitation in the optimal range between CM-lysozyme and its homologous antiserum, as described previously (Gerwing and Thompson, 1968). The control system used was native lysozyme and its homologous antiserum. All tests were done in triplicate, and the per cent inhibition was calculated as the percentage of protein precipitated in test samples in comparison to the amount precipitated in controls containing just antiserum and CM-lysozyme, which were done simultaneously. The haptenic activity of the synthesized NH_2-terminal decapeptide was measured by estimating its ability to inhibit complement fixation between CM-lysozyme and its homologous antiserum as described previously (Gerwing and Thompson, 1968). The negative control used in this instance was performic acid oxidized ferredoxin and its homologous antiserum.

Edman Degradation. Samples of T-11 to be subjected to Edman degradation were first desalted by passage through

* From the Department of Microbiology, The University of British Columbia, Vancouver 8, B.C., Canada. *Received January 21, 1970.* This work was supported in part by an H. R. McMillan Student Scholarship and by the Medical Research Council of Canada.

† Present address: Laboratory of Medical Entomology, Kaiser Foundation Research Institute, San Francisco, Calif.

Sephadex G-25 columns (2.5 × 40.0 cm) which had been equilibrated with distilled water. The eluted peptide was lyophilized and used as the starting material for the degradation steps. The methods outlined by Konigsberg and Hill (1962) and Konigsberg (1967) were used with minor modifications. The peptide (1.5 μmoles) was dissolved in 0.3 ml of 50.0% aqueous pyridine containing 2.0% trimethylamine. Enough phenyl isothiocyanate (Eastman Kodak) was added to yield a 50-fold molar excess of the coupling reagent. The reaction was allowed to take place for 2.0 hr at 45° in a sealed container which had been purged with nitrogen.

Excess reagent was subsequently removed by first diluting the reaction mixture to 1.0 ml with water and then by extracting twice with 1.0 ml of benzene. The coupled peptide was dried under vacuum and cyclization was subsequently carried out by treatment with 0.3 ml of anhydrous trifluoroacetic acid for 30 min at 45° under nitrogen. The trifluoroacetic acid was removed by evaporation of the mixture over P_2O_5 at 25°. The residue was dissolved in 0.2 ml of 0.2 M acetic acid and extracted twice with 1.0 ml of benzene. The remaining peptide was again dried under vacuum and dissolved in 0.1 ml of 0.2 M acetic acid and adsorbed onto a small Dowex 50 column (4.0 × 50.0 mm) which was in the pyridine form and had been equilibrated with 0.2 M acetic acid. Ten milliliters of 0.2 M acetic was used first as eluent, followed by elution of the peptide with 5.0 ml of pyridine acetate buffer at pH 5.0 (2.0 M in acetic acid). The acetic acid wash was discarded and the material eluting with the buffer was lyophilized and redissolved in 0.3 ml of aqueous pyridine containing 2.0% trimethylamine. This represented the completion of one cycle of degradation. Four 10-μl aliquots, each one containing approximately 0.05 μmole of degraded T-11, were removed from the degraded material. One aliquot was used for amino acid analysis and the other three were used for hapten inhibition experiments.

Two further cycles were carried out, the only difference being that the volumes taken after the second and third cycle were 20.0 and 30.0 μl, respectively, to compensate for losses of peptide during extraction and chromatography. Also, each time a cycle was completed, the peptide was taken up in less aqueous pyridine to compensate for the quantities removed after previous steps and to maintain the concentration at the same level. The samples used for amino acid analysis were dried, redissolved in 6.0 N HCl, and hydrolyzed. This was done before immunological testing on the other three aliquots in order to determine the exact amount of peptide in each assayed sample. The samples used for hapten inhibition tests were dried and washed three times with distilled water to remove the pyridine and dissolved in 0.1 ml of 0.9% NaCl. Hapten inhibition tests were carried out concurrently with positive controls containing 0.04, 0.02, and 0.01 μmole of T-11, in order to cover the range found to be present in the degraded samples.

Leucine Amino Peptidase Digestion of T-11. The leucine amino peptidase was obtained from Worthington Biochemical Co., and was assayed for activity before use. The experimental procedures are outlined below and are similar to those recommended by Light (1967).

EXPERIMENTAL SYSTEM. Peptide T-11 (8.0 mg, 3.2 μmoles) was dissolved in 1.0 ml of 0.14 M trimethylamine acetate buffer at pH 8.5 containing 0.002 M $MgCl_2$. At zero time, 0.25 mg (0.3 ml of the activated enzyme preparation containing 25.0 units of enzyme activity) of leucine amino peptidase was added.

SUBSTRATE CONTROLS. Peptide T-11 (1.0 mg, 0.4 μmole) was dissolved in 0.125 ml of the trimethylamine acetate buffer. Peptide T-11 (4.0 mg, 1.6 μmoles) was dissolved in 0.5 ml of the trimethylamine acetate buffer. At zero time, 0.125 mg (0.15 ml of the activated enzyme preparation) of heat-inactivated leucine amino peptidase (boiled for 10 min) was added.

ENZYME CONTROL. The activated enzyme preparation (0.125 mg, 12.5 units) of leucine amino peptidase was dissolved in 0.5 ml of the trimethylamine acetate buffer.

All the tubes were incubated at 37° and samples of 0.1 ml were taken from the test, the enzyme control, and substrate control b at 8, 16, 24, 40, and 48 hr. A sample was also taken from the test solution at 32 hr, just before the addition of fresh enzyme in the same amounts as that used initially. Heat-inactivated enzyme and active enzyme were also added at this time to the appropriate substrate controls in the same manner. As the samples were taken at the various times, the pH was lowered by the addition of 0.9 ml of 0.1 N acetic acid, in order to terminate the enzyme reaction. Amino acid analyses were carried out on 0.3 ml of each of the test samples. This material was taken to dryness and dissolved in the pH 2.2 starting buffer and quantitative amino acid analysis was carried out in order to determine the extent of enzyme degradation. A comparable sample was taken from the enzyme control and analyzed to determine the rate of release of amino acids from the enzyme itself. Under these conditions, 0.004 μmole of leucine was detected when the enzyme alone was incubated for 48 hr. Correction was made for this amount in calculating the degree of digestion in the experimental samples.

When the analyses had been carried out on each digest sample and the extent of enzyme cleavage was estimated, hapten inhibition tests were carried out in triplicate on 0.1-ml samples of each aliquot (these were found to contain 60 μg of T-11 after amino acid analysis of other aliquots). Hapten inhibition tests were also carried out on the boiled enzyme controls sampled at 8, 16, and 48 hr and on the enzyme controls taken at 8, 24, and 48 hr. The T-11 control was tested only at 48 hr to determine the effect of prolonged incubation at 37° on its haptenic activity.

Carboxypeptidase Digestion. Carboxypeptidases A and B (Worthington CPA, DFP treated, and CPB, not DFP treated) were assayed for activity according to the method of Folk and Schirmer (1963), and Folk *et al.* (1960), respectively, prior to use. The methods used for the digestion of T-11 were basically those described by Young *et al.* (1966). In this experiment, 3.0 ml of T-11 containing 0.75 μmole was dissolved in 0.9% NaCl, and 3.0 ml of 0.9% NaCl was used as a control. The pH was adjusted to 8.0 with 2% $NaHCO_3$, and 0.01 ml of carboxypeptidase B (100 μg containing 0.4 unit of activity) was added to each tube, and reaction was allowed to proceed for 60 min at 37°. Then 0.01 ml of carboxypeptidase A (250 μg containing 23 units of activity) and 0.1 ml of 10.0% LiCl were added to both tubes, and incubation at 37° was continued. Additional aliquots of 0.01 ml of carboxypeptidase A were added at 2, 15, and 24 hr to both tubes. Samples of 0.4 ml were taken from both test and control tubes at 2, 6, 15, 24, and 40 hr. From these, 0.1 ml was dried and taken up in starting buffer (pH 2.2) for amino acid analysis. The remainder was used for hapten inhibition tests.

TABLE I: Quantitative Amino Acid Analysis of Aliquots Taken after Three Cycles of Edman Degradation.

Amino Acid	Residues in T-1 1	Step I		Step II		Step III	
		μmole Present	μM Ratio	μmole Present	μM Ratio	μmole Present	μM Ratio
CM-cysteine	3	0.212	3.0	0.036	3.3	0.020	1.8[a]
Aspartic acid	4	0.217	3.1[a]	0.033	3.0[a]	0.031	2.8[a]
Threonine	1	0.068	0.97	0.013	1.2	0.012	1.1
Serine	4	0.261	3.7	0.042	3.8	0.040	3.6
Proline	1	0.097	1.4	0.016	1.5	0.015	1.4
Alanine	3	0.242	3.5	0.035	3.2	0.037	3.4
Valine	1	0.077	1.1	0.012	1.1	0.014	1.3
Isoleucine	2	0.151	2.2	0.022	2.0	0.022	2.0
Leucine	3	0.222	3.2	0.025	2.3[a]	0.024	2.2[a]
Sample size analyzed (μl)		10.0		20.0		30.0	
μmole present		0.070		0.011		0.011	

[a] Amino Acids removed after each step.

Peptide Analyses. Quantitative estimations, using a Beckman Model 120 amino acid analyzer, of the digestion products of T-11 by leucine amino peptidase, and carboxypeptidases A and B were based on the calculation of the free amino acids released by digestion. Analyses of T-11 and T-11 following Edman degradation were performed after acid hydrolysis in 6 N HCl at 108° for 18 hr.

Solid-Phase Peptide Synthesis. The following peptide (comprising the NH_2-terminal decapeptide of T-11) was synthesized by the solid-phase method described originally by Merrifield (1964): H-Asn-Leu-CM-Cys-Asn-Ile-Pro-CM-Cys-Ser-Ala-Leu-OH. The modification of the original methods and precise procedures recommended by Stewart and Young (1969) were used throughout. The chloromethylated copolysterene divinylbenzene resin was obtained from Bio-Rad and the *t*-Boc-amino acids were obtained from Sigma Chemical Co.

Synthesis was carried out on 2.0 g of resin, and amino acids were reacted at each step in a 3.5 molar excess, with the exception of asparagine (in the form of *p*-nitrophenyl ester), in which a 5-fold excess was used. Cysteine and serine were added as the *S*-benzyl and *O*-benzyl derivatives, respectively, and cleavage of the peptide was carried out on an HF line in order to ensure deprotection of the cysteine residues.

The peptide was alkylated with iodoacetate and purified chromatographically according to the procedures used in the preparation of the T-11 peptide (Gerwing and Thompson, 1968).

Results

Quantitative estimation of peptide material remaining after each cycle of Edman degradation demonstrated that efficient removal of the NH_2-terminal amino acids had been achieved, but that considerable amounts of peptide were being lost during each step. The results of the amino acid analyses made on 10.0-, 20.0-, and 30.0-μl samples taken after the first three steps are shown in Table I. Studies on the ability of these samples to inhibit precipitation between CM-lysozyme and its homologous antiserum were subsequently carried out and compared for efficiency to various amounts of T-11. The results are summarized in Table II and represent the averaged values of tests run in triplicate. It can be seen that a very marked reduction in the haptenic activity of T-11 was noted after the removal of the first amino acid from the NH_2 terminus and that removal of the following two amino acids resulted in total loss of haptenic activity.

Because it was possible that the loss of haptenic activity might be due to the relatively rigorous treatment involved in Edman degradation, another experiment was designed using leucine amino peptidase to remove the NH_2-terminal amino acids. Digestion was continued for 48 hr with addition of fresh enzyme at 32 hr. Samples were taken at 8-hr intervals over the 48-hr period and aliquots were analyzed to determine the degree of digestion. Correction was made for the 0.004

TABLE II: Inhibition of Precipitation between CM-lysozyme and Its Homologous Antiserum by T-11 and the T-11 and the Three Products of Edman Degradation.

Sample	Quantity (μmole)	Ppt *A* at 280 mμ	% Inhibn
T-11	0.04	0.063	56.6
T-11	0.02	0.067	53.9
T-11	0.01	0.073	49.7
T-11-1[a]	0.07	0.118	18.6
T-11-2	0.011	0.143	0.0
T-11-3	0.011	0.167	0.0
Saline control		0.145	0.0

[a] Numbers 1, 2, and 3 correspond to removal of the first, second, and third amino acid.

TABLE III: Percentages of Amino Acids Released during Leucine Amino Peptidase Digestion of T-11[a] (Relative to the Micromolar Concentration of T-11 (0.074 μmole) Present in the Samples).

Amino Acid	Digestion Time									
	8 hr		16 hr		24 hr		32 hr		40 hr		48 hr	
	μmole	% Cleavage	μmole	% Cleavage	μmole	% Cleavage	μmole	% Cleavage	μmole	% Cleavage	μmole	% Cleavage
CM-cysteine	0.0124	16.8	0.028	37.8	0.047	63.5	0.052	70.3	0.056	75.7	0.118	160.0
Asparagine	0.016	22.0	0.028	37.8	0.043	58.1	0.054	73.0	0.098	133.0	0.149	201.4
Serine											0.015	20.3
Proline											0.025	33.8
Isoleucine											0.081	109.5
Leucine	0.022	29.8	0.037	50.0	0.057	77.0	0.058	78.5	0.057	77.0		

[a] Sequence of T-11: H^{74}-Asn-Leu-CM-Cys-Asn-Ile-Pro-CM-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CM-Cys-Ala-Lys^{96}-OH.

TABLE IV: Measurement of the Ability of T-11 and Samples at Various Stages of Leucine Amino Peptidase Digestion to Inhibit the Precipitation of CM-lysozyme with Its Homologous Antiserum.[a]

Sample	Sampling Times	Ppt A at 280 mμ	% Inhibn
T-11 digests	8	0.150	46.6
	16	0.165	41.3
	24	0.196	30.0
	32	0.206	26.5
	40	0.208	26.0
	48	0.218	22.4
Boiled enzyme + T-11	8	0.147	47.7
	16	0.152	45.9
	24	0.135	52.0
	48	0.159	43.4
Enzyme control (no hapten)	8	0.276	1.8
	24	0.278	1.0
	48	0.281	0.0
Hapten control (60 μg of T-11)	48	0.146	48.0
Saline control	0	0.281	0.0

[a] All tests were performed using comparable concentrations of T-11.

μmole of leucine released from the enzyme control. Per cent cleavage of the residues was calculated as a percentage of the amount of free amino acid from the amount of T-11 present in the sample. The results shown in Table III demonstrate that total removal of the first four amino acids (Asn-Leu-Cm-Cys-Asn) was achieved after 48 hr, and that appreciable quantities of the next three (Ile-Pro-CM-Cys) had also been removed.

Samples containing 0.025 μmole (60 μg) of T-11 were taken from all the digest aliquots and controls and tested in triplicate for their capacity to inhibit precipitation between CM-lysozyme and its homologous antiserum. The results are shown in Table IV. These data substantiate to some extent the observations made in the experiments on the Edman degradation of T-11, in that the NH_2-terminal portion of the peptide appeared to play a critical role in its haptenic activity. Samples of the digests taken at 24, 32, and 48 hr were tested for haptenic activity over a range of 50.0–1.0 μg. Boiled enzyme control samples taken at 24 and 48 hr were also assayed in this manner. The results are shown in Figure 1, and confirm that the digest samples have lost much of their ability to inhibit the precipitation between CM-lysozyme and its antiserum.

Carboxypeptidase experiments were carried out to determine the effect of the COOH-terminal residues on the haptenic activity of T-11. Amino acid analyses of the digest showed that 80.0–90.0% of the two COOH-terminal residues (Ala-Lys)

TABLE V: Amino Acid Composition and Molar Ratio of the NH_2-Terminal Decapeptide of T-11 Prepared by the Solid-Phase Synthetic Method.[a]

Amino Acid	μmole	M Ratio	Residues
Aspartic acid	0.079	1.41	2
Serine	0.051	0.91	1
Proline	0.062	1.10	1
Alanine	0.055	0.98	1
Isoleucine	0.058	1.04	1
Leucine	0.109	1.95	2
CM-Cys	0.077	1.38	2

[a] The sequence of the peptide is H-Asn-Leu-CM-Cys-Asn-Ile-Pro-CM-Cys-Ser-Ala-Leu-OH.

were removed after 40-hr digestion but that no significant levels of other amino acids were removed since the third amino acid in from the COOH terminal is CM-Cys, which is known not to be readily attacked by carboxypeptidase A. Hapten inhibition studies on the 40-hr digest samples showed that the removal of these two residues had no effect on the ability of T-11 to inhibit precipitation between CM-lysozyme and its antiserum.

The specificity of both T-11 and the various samples under test was determined using native lysozyme and its homologous antiserum. At no time did any of the preparations demonstrate the capacity to inhibit this reaction.

The data presented above strongly indicated the participation of the NH_2-terminal region of T-11 in its haptenic activity. The NH_2-terminal decapeptide of T-11 was synthesized by the solid-phase method, and alkylated and purified according to previously described methods (Gerwing and Thompson, 1968). The molar ratios and expected amino acid composition of the synthesized peptide are shown in Table V. When the purity of the peptide was tested by high-voltage paper electrophoresis (Mitchell and Gerwing Levy, 1970), only one ninhydrin-positive spot was observed. It was concluded that only one major peptide was present.

The ability of the synthesized decapeptide to exert haptenic activity was determined by testing its ability to inhibit complement fixation between CM-lysozyme and its homologous antiserum. Similar tests on T-11 were run at the same time. The results are presented in Figure 2 and show that the NH_2-terminal decapeptide exhibited considerably more inhibitory activity on the system than did T-11. The specificity of this inhibitory behavior was demonstrated by showing that neither the decapeptide nor T-11 inhibited complement fixation between oxidized ferredoxin and its homologous antiserum.

Discussion

The data obtained from experiments involving both Edman degradation and leucine amino peptidase digestion of T-11 implied the importance of the NH_2-terminal region of this peptide in its haptenic activity. Although these two experiments demonstrated qualitatively the same result, they differed quantitatively. When T-11 was degraded sequentially

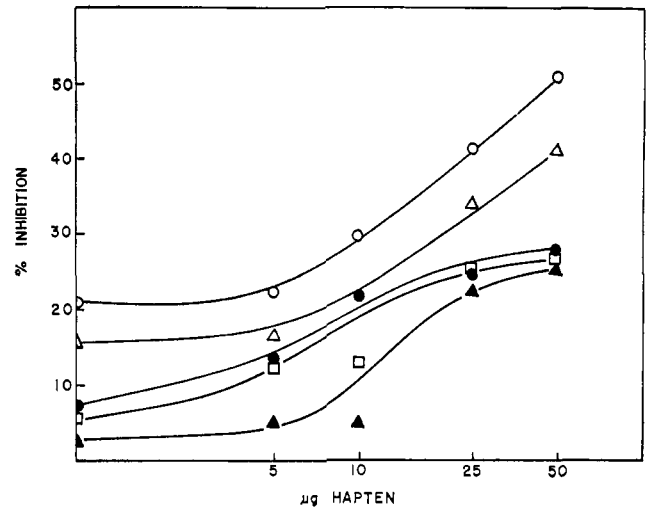

FIGURE 1: Inhibition of precipitation between CM-lysozyme and its homologous antiserum by leucine amino peptidase digested T-11 and controls; (○-○) 24-hr incubation in the presence of boiled enzyme (control); (△-△) 48-hr incubation in the presence of the boiled enzyme (control); (●-●) 24-hr digest; (□-□) 32-hr digest; (▲-▲) 48-hr digest.

by the Edman technique, it appeared that the removal of the two NH_2-terminal residues, Asn-Leu, totally destroyed its haptenic properties. On the other hand, after the T-11 peptide had been digested for 48 hr with leucine amino peptidase, and virtually all of the first four amino acids (Asn-Leu-CM-Cys-Asn) and a small amount of the next three (Ile-Pro-CM-Cys) had been removed, the digests retained a considerable amount

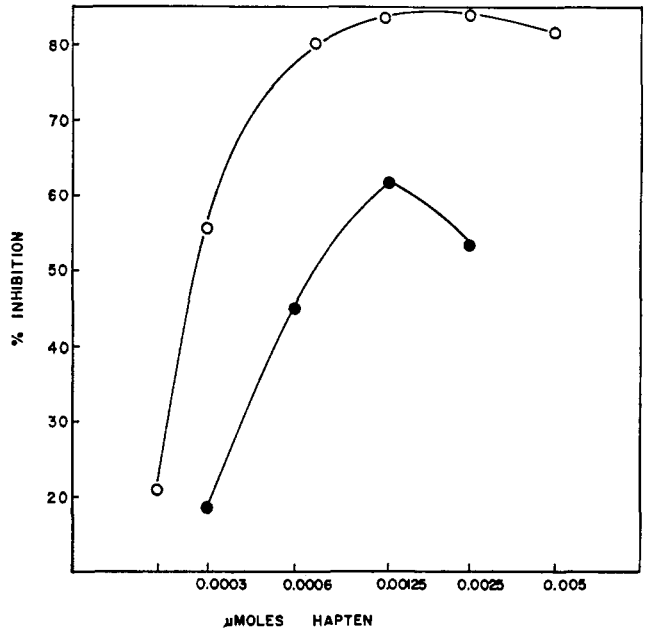

FIGURE 2: The inhibition of complement fixation between CM-lysozyme and its homologous antiserum when tested in the presence of T-11 (●-●) and the NH_2-terminal decapeptide of T-11 (○-○) at various concentrations. The complement dilution at which the tests were performed was 1:175.

of inhibitory activity, especially at the high concentrations (Figure 1). This was probably because small amounts of undigested T-11 remained in the digests, and since T-11 is capable of causing in the region of 20% inhibition of precipitation at levels as low as 1.0 μg (Figure 1), it would take only very small amounts to alter the apparent degree of inhibition caused by these digests.

The experiment with carboxypeptidases A and B was repeated, and in both instances, only the two COOH-terminal amino acids were released, and this apparently had no effect on the haptenic activity of T-11.

The probability that the NH_2-terminal region of T-11 was involved in the peptide's haptenic activity was confirmed by the synthesis and immunological testing of the NH_2-terminal decapeptide of T-11 which comprised the sequence Asn^{74}–Leu^{83} (Asn-Leu-CM-Cys-Asn-Ile-Pro-CM-Cys-Ser-Ala-Leu). It was somewhat surprising to note that the decapeptide exhibited more potent haptenic activity than did T-11 when this property was determined by measuring the ability of both preparations to inhibit complement fixation between CM-lysozyme and its homologous antiserum (Figure 2).

These findings are particularly interesting when they are compared with those of Arnon and Sela (1969) on native lysozyme. The sequence designated the "loop" peptide by these workers comprises the sequence Cys^{64}–Leu^{83} and was shown to be an antigenic determinant in the native lysozyme molecule. The NH_2-terminal decapeptide of T-11 (Asn^{74}–Leu^{83}) falls within the sequence encompassed by the loop peptide. Since no cross-reactivity can be demonstrated between T-11 and its degradation products, and the native lysozyme immune system, this overlapping haptenic region may be coincidental. However, the data emerging from studies on lysozyme and its alkylated derivative present interesting models for studying the relationship between conformation and amino acid sequence in the characterization of antigenic determinants.

Acknowledgment

The authors thank Dr. J. D. Young, the Laboratory of Medical Entomology, Kaiser Research Foundation Research Institute and Allergy Research Division, Kaiser Foundation Hospitals, San Francisco, for her advice and cooperation in performing the HF cleavage of the decapeptide used in this work.

References

Arnon, R., and Sela, M. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 163.

Canfield, R. E. (1963), *J. Biol. Chem. 238*, 2691.

Canfield, R. E., and Anfinsen, C. B. (1963), *J. Biol. Chem. 238*, 2684.

Folk, G. E., Piez, K. A., Carroll, W. R., and Gladner, J. (1960), *J. Biol. Chem. 235*, 2272.

Folk, G. E., and Schirmer, E. H. (1963), *J. Biol. Chem. 238*, 3884.

Fujio, H., Imanishi, M., Nishioka, K., and Amano, T. (1968), *Biken J. 11*, 207.

Gerwing, J., and Thompson, K. (1968), *Biochemistry 7*, 3888.

Konigsberg, W. (1967), *Methods Enzymol. 11*, 461.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.

Light, A. (1967), *Methods Enzymol. 11*, 426.

Merrifield, R. B. (1964), *Biochemistry 3*, 1385.

Mitchell, B., and Gerwing Levy, J. (1970), *Biochemistry 9*, 1839.

Shinka, S., Imanishi, M., Miyagawa, M., Amano, T., Inouye, M., and Tsugita, A. (1967), *Biken J. 10*, 89.

Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.

Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966), *Biochemistry 5*, 1482.

Young, J. D., and Leung, C. Y. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 326.