Studies on the Antigenic Properties of Egg-White Lysozyme. I. Isolation and Characterization of a Tryptic Peptide from Reduced and Alkylated Lysozyme Exhibiting Haptenic Activity*

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ABSTRACT: The haptenic properties of tryptic peptides from *S*-carboxymethylated lysozyme have been investigated. Hapten inhibition studies have been carried out using precipitation and complement fixation inhibition as test systems. Both whole tryptic digests and peptides prepared and isolated by the method of Canfield (Canfield, R. E. (1963a), *J. Biol. Chem. 8*, 2691) have been investigated.

A single peptide (T-11) was found to exhibit haptenic properties which on a weight basis inhibited test systems at least twice as strongly as tryptic digests, whereas none of the other peptides isolated exhibited detectable antigenic properties. Peptide T-11 has the amino acid sequence: NH_2-Asn-Leu-CMC-Asn-Ile-Pro-CMC-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CMC-Ala-Lys. The relationship between this peptide and antigenic determinants in native lysozyme is discussed.

Relatively few proteins have been studied definitely with respect to the regions of the molecules responsible for antigenic specificity. Thus far, oxidized ribonuclease (Brown, 1962), sperm whale myoglobin (Crumpton and Wilkinson, 1965), tobacco mosaic virus protein subunit (Benjamini *et al.*, 1964), and native lysozyme (Shinka *et al.*, 1967) have been characterized in this way.

The work of Benjamini *et al.* (1964) on TMVP has been the most extensive to date, in which he showed strong antigenic properties initially in a tryptic peptide (T-8) of 20 residues, and later found that the five amino acids at the C terminal of this peptide were critical for antigenicity (Young *et al.*, 1966).

Shinka *et al.* (1967) have recently published evidence that in native lysozyme, four peptic peptides exhibited antigenic properties and that all of these contained the sequence from Gln^{57} to Ala^{107}, and that they differed only in their cleavage points in the loop between Cys^{80} and Cys^{94}. In this study CM-lysozyme[1] has been used as the antigen. In initial experiments we found that CM-lysozyme did not cross react at the precipitin level with native lysozyme antiserum, and none of the tryptic peptides isolated from it showed any capacity to act as haptens with the native system. We felt that a comparative study might throw some light on the interrelated role of primary and tertiary structure in proteins in determining antigenicity. We have successfully isolated a single tryptic peptide from CM-lysozyme which exhibits strong haptenic activity. The peptide encompasses the sequence Asn^{74} to Lys^{96} which falls within the sequence isolated by Shinka *et al.* (1967) and agrees with their findings using tryptic digests of native lysozyme. The implications of these observations are discussed.

Experimental Procedures

Immunization Procedure. Egg-white lysozyme (Worthington Biochemical Corp.) was used throughout the experiments. Reduction and alkylation with iodoacetic acid were carried out according to the procedure described by Canfield and Anfinsen (1963). Treated lysozyme (30 mg) was suspended in 3.0 ml of complete Freund's adjuvant (Difco). Three rabbits were immunized by the administration of the material as follows. The emulsion (0.2 ml) was injected into a footpad of each of the four extremities, and 0.2 ml was administered intramuscularly in the left hind leg. The following day 1.0 ml of alum-precipitated antigen (prepared according to the method described by Kabat and Mayer, 1961) containing 1.5 mg of the antigen was injected intravenously into each animal. From 4 weeks on, the antibody titer was found to be stable. Ear bleeds were made at weekly intervals, and the serum was stored in 50.0-ml batches at 4°. Merthiolate at a final concentration of 1:10,000 was added as a preservative.

Quantitative Precipitin Reaction. Initial studies on the antigenic properties of reduced and carboxymethylated lysozyme were carried out with the use of the immune precipitation reaction. Optimal proportions were determined by incubating varying amounts of antigen with constant amounts of antiserum at 37° for 1 hr followed by 18 hr at 4°. The precipitates were centrifuged at 2000*g*, washed twice with 0.9% NaCl, and dissolved in 1.0 ml of 0.1 N NaOH. Absorbance at 280 mμ was read on a Beckman DBG spectrophotometer. The sera from the three rabbits were all titered against a stock solution of CM-lysozyme containing 100 μg/ml in 0.9% NaCl.

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: CM-lysozyme, *S*-carboxymethylated lysozyme.

Sera 1 and 2 were identical in titer and precipitated optimally with 25 μg of CM-lysozyme/ml of serum. Serum 3 reacted optimally with 50 μg of CM-lysozyme under the same conditions, and was selected for use in any extensive precipitin testing because of its higher titer.

Hapten inhibition studies with tryptic peptides were performed as follows. The peptide (0.1 ml) in 0.9% NaCl was mixed with 0.4 ml of undiluted serum and incubated at 37° for 1 hr and stored for 18 hr at 4°. Antigen was then added at the calculated amount to yield optimal precipitation with 0.4 ml of antiserum, and the incubation at 37 and 4° was repeated. Resulting precipitates were then prepared as described above and read for 280-mμ absorbance. The per cent inhibition was calculated as the percentage decrease in optical densities at 280 mμ, taking the saline control as 100%.

Complement Fixation Reaction. Freeze-dried guinea pig serum (3.0-ml quantities) was used as a source of complement. The serum was reconstituted for each test in distilled water, and dilutions were made between 1:25 (this having previously been calculated as a suitable initial dilution) and 1:250 in Veronal-buffered saline prepared according to the method of Brooksby (1952). This buffer was used for all dilutions of antigen, haptens, and antiserum throughout complement fixation testing. A pooled sample of antiserum and a stock solution of CM-lysozyme containing 100 μg/ml were used throughout. Complement, antigen, and antiserum (each in 1.0-ml volumes) were mixed and incubated at 4° overnight. A 2% suspension of sheep erythrocytes in Veronal-buffered saline was sensitized by mixing them with an equal volume of a 1:50 dilution of the recommended standard solution of hemolysin (Difco-Bacto antisheep hemolysin) for 10 min. This preparation (1 ml) was mixed with the test series and incubated for 30 min at 37°. The tubes were shaken once after 15 min. Unhemolysed cells were removed by centrifugations at 1800*g* and the supernatants were read on a Klett colorimeter using a green filter. End points were calculated as 50% hemolysis on a probit plot of actual percentages of the Klett readings according to the method described by Wright (1963). The pooled test serum titered at a 1:80 dilution. Titration of the serum had been carried out using a standard dose of CM-lysozyme (0.4 μg in 1.0 ml). The antigen was titrated by repeating the tests using 1:80 serum throughout with varying amounts of antigen. The data showing the calculation of the optimal antigen range are shown in Figure 1.

Hapten inhibition studies involved the use of standard amounts of antigen (0.8 μg) and antiserum (1:80) and varying amounts of peptide and complement. To 1.0-ml aliquots of 1:80 antiserum, 0.2 ml of test peptides was added. When initial screening tests were carried out, the peptides were all added at a concentration of 50 μg. Once inhibition was observed only in two preparations, the positive samples were assayed over a range of 50.0–0.01 μg. The peptide–antiserum mixtures were incubated at 37° for 1 hr and for 18 hr at 4°. Subsequently, 0.8 μg of antigen in 1.0 ml, and 1.0 ml of complement containing dilutions between 1:40 and 1:125 of the guinea pig serum, were added and the mixture was left at 4° overnight. On day 3 the hemolysin system was added as described above, and the end point was again taken as 50% hemolysis.

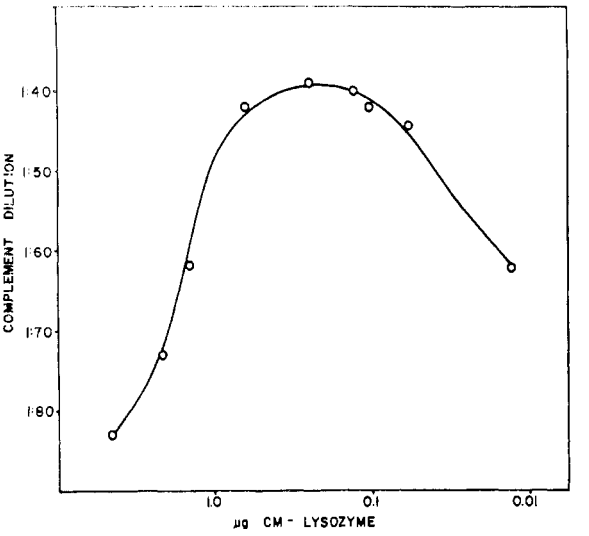

FIGURE 1: Titration curve showing the optimal range of CM-lysozyme to yield 50% hemolysis in standard complement fixation reactions.

Preparation of Tryptic Peptides. The carboxymethylated lysozyme was digested with trypsin (Worthington lyophilized containing 125 units/mg) as described by Canfield (1963a). Digests usually lacked the ability to precipitate with homologous antiserum but the precaution was taken of passing them through an ultrafilter designated to exclude only material of less than 10,000 molecular weight (Amicon ultrafilter, UM-1 filter pad). In the initial stages of the work, Canfield's method for peptide separation, using a Dowex 50-X4 column at 35° with an eight-chambered gradient system, was employed with each buffer at a volume of 500 ml. Each fraction thus separated was dried on a flash evaporator at 45°, washed with distilled water, freeze dried, and dissolved or resuspended (some peptides were only sparingly soluble) in 0.9% NaCl.

When our initial studies indicated strong haptenic activity in the peak containing the T-11 peptide (Canfield's nomenclature), and the concomitant lack of haptenic behavior on the part of any other peptide fraction, the method was somewhat simplified for further separations. A four-chambered system was set up containing the first four pyridine–acetate buffers recommended by Canfield, ranging from 0.1 M (at pH 3.80) to 0.4 M (at pH 4.47). Volumes of 500 ml for each buffer were used and the peptides were eluted through the Dowex 50-X4 column at 35° until 1 l. had been collected. At that time, the remaining adsorbed peptides were eluted by running 2.0 M buffer at pH 5.18 through the column. Fractions of 10.0 ml were collected from which 0.3-ml aliquots were analyzed by ninhydrin after alkaline hydrolysis according to the method of Hirs *et al.* (1956). A representative elution profile is shown in Figure 2. The method permitted the elution of the T-11 peptide in a state free from other peptides in a sharply delineated peak. Amino acid analysis of this peak established its identity with Canfield's T-11 peptide.

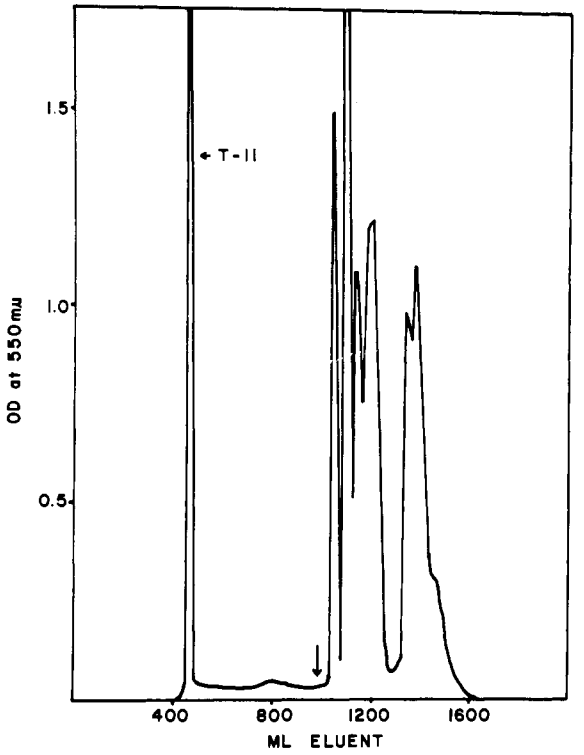

FIGURE 2: Elution profile of tryptic peptides of CM-lysozyme. Ninhydrin reactions were carried out after alkaline hydrolysis. Arrow designates change to 2.0 M buffer at pH 5.18.

Protein and Peptide Analysis. Quantitative estimation of CM-lysozyme was calculated initially by the Lowry method (Lowry *et al.*, 1951) and equated on a weight basis with its extinction at 280 mμ. Subsequently, estimations were made on a spectrophotometric basis. Quantitation of the T-11 and other peptides was calculated initially on a dry weight basis. Closer estimations of the T-11 peptide involved the calculation of micromoles present obtained from quantitative amino acid analysis of the peptide. Amino acid analysis was carried out according to the method of Spackman *et al.* (1958) on a Beckman Model 120 amino acid analyzer.

Experimental Results

Initial screening for haptenic activity involved the mixing of duplicate samples of 0.4 ml of titered antiserum with 0.1 ml of the various peptide fractions (containing between 200 and 1000 μg per ml depending upon solubility) dissolved in 0.9% NaCl, and incubating the mixture at 37° for 1 hr followed by 18 hr at 4°. Saline controls in triplicate were done simultaneously. The following day, 10 or 20 μg of CM-lysozyme in 0.3 ml of 0.9% NaCl (depending upon the serum being used) was added, and the incubation was repeated. The precipitates formed were washed in 0.9% NaCl, dissolved in 1.0 ml of 0.1 N NaOH, and read for 280-mμ absorbance. These experiments were repeated, using sera from the three individual animals, and in each case, the only peptide fraction showing evidence of hapten inhibition was that which correlated with the T-11 peak from the Dowex column. In order to establish that our fraction was in fact the same as that described by Canfield, an amino acid analysis was carried out on it. The results are shown in Table I and correlate well with Canfield's data. The preparation was virtually free of all other amino acids except for trace quantities of Glu and Gly which were present in amounts of less than 0.002 μmole. Ring tests on this fraction proved to be negative, showing that no precipitable material was present. The stock solution of this peptide used for most of the the tests reported here contained 500 μg/ml.

TABLE I: Amino Acid Analysis of Peptide T-11.[a]

Amino Acid	1[b]	2[b]	Residues
Lysine	0.27	0.0211	1
S-CM-cysteine	0.73	0.0597	3
Aspartic acid	1.11	0.0722	4
Threonine	0.27	0.0195	1
Serine	1.03	0.0618	4
Proline	0.28	0.0154	1
Alanine	0.78	0.0516	3
Valine	0.25	0.0173	1
Isoleucine	0.55	0.0341	2
Leucine	0.82	0.0559	3

[a] The micromolar ratios are in agreement with those determined by Canfield (1963a) and shown in column 1. [b] In micromoles.

A quantitative hapten inhibition test was carried out on T-11 using between 50 and 0.1 μg of the peptide and compared on a weight basis with a similar test on the total tryptic digest. Serum from rabbit 3 was used since this had a slightly higher precipitating titer than the other two sera. The tests were run in duplicate and the results (using the mean values) are shown in Figure 3. Subsequent testing showed that these results were closely reproducible and that variation for each point lay between 1.5 and 2.0%. It was found that 25 μg of T-11 yielded maximum inhibition of about 60% and that amounts greater than this did not increase the degree of inhibition.

In order to show that the inhibition observed here was not unique for precipitation, tests for inhibition of complement fixation were also employed. The antiserum from rabbit 1 was used which titered at 1:80, with optimum antigen levels falling between 0.1 and 0.8 μg (Figure 1). In testing for hapten inhibition, 0.8 μg of CM-lysozyme was used standardly in order to work in a region of slight antigen excess. The initial test was carried out on T-11 and the total digest employed only two dilutions of complement (1:42 and 1:50) since these had previously been calculated as the 50% hemolysis end points for the reaction of the antigen and antibody. Under these conditions, total inhibition was observed for the T-11 peptide between 50 and 10 μg, and between 50 and 25 μg for the tryptic digest. In order to determine the actual degree of

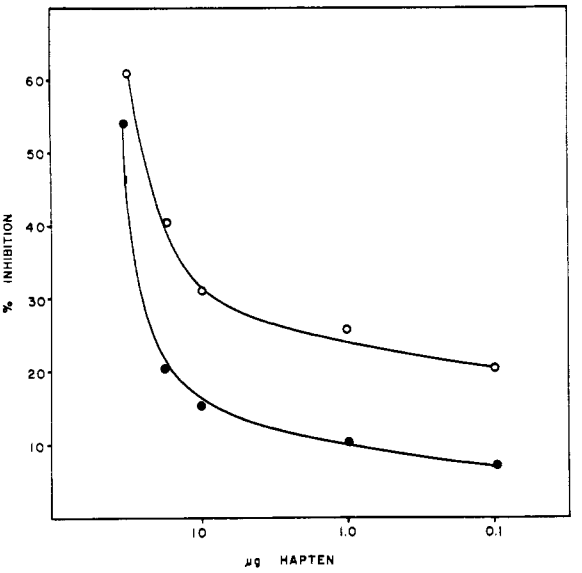

FIGURE 3: Inhibition of immune precipitation by peptide T-11 (○—○) and the total tryptic digest (●——●) at varying concentrations.

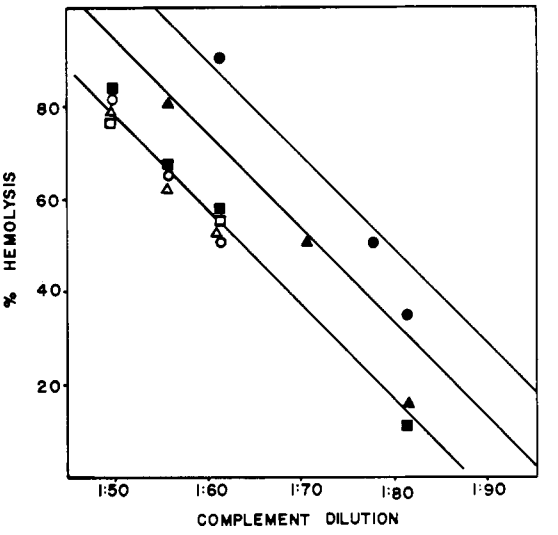

FIGURE 5: Inhibition of complement fixation with CM-lysozyme and homologous antiserum by the total tryptic digest at varying concentrations; ●—●, 50 μg; ▲—▲, 25 μg; ■—■, 10 μg ○—○, 1.0 μg; △—△, 0.1 μg; and □—□, no hapten control.

inhibition, the succeeding test was expanded to cover a broader range of complement and hapten levels. Tests were run concurrently on the other tryptic peptides which had failed to show inhibition of the precipitation reaction; these were tested at only one level which varied between 50 and 200 μg depending upon the maximum solubility of the particular peptide. As in the previous tests, the only fractions showing inhibition were the total digest and T-11. The results for these two preparations are documented in Figures 4 and 5, and are correlated in Figure 6, in which, on a weight basis, peptide T-11 showed considerably more inhibitory capacity than the tryptic digest, demonstrating detectable inhibition in concentrations as low as 1.0 μg whereas the total digest showed only slight inhibition at 10 μg.

The final control measure in these experiments tested the specificity of the inhibition reaction observed with the T-11 peptide. A system of native lysozyme and its homologous antiserum were used for this purpose. The antiserum had been prepared and titered in an identical manner as that described here for the CM-lysozyme antisera. We had previously noted that antiserum

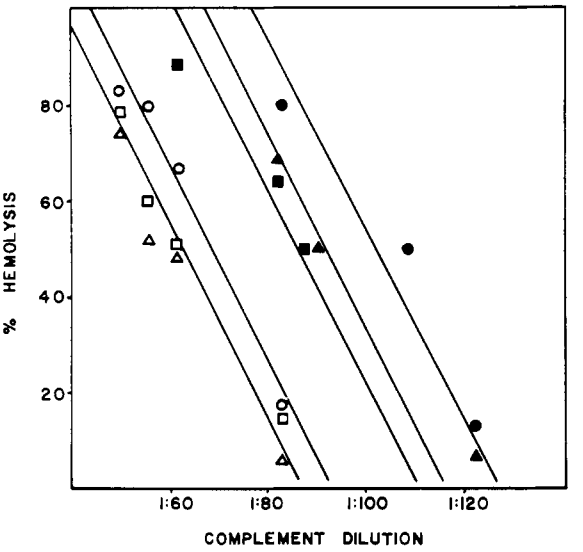

FIGURE 4: Inhibition of complement fixation with CM-lysozyme and homologous antiserum by peptide T-11 at varying concentrations; ●—●, 50 μg; ▲—▲, 25 μg; ■—■, 10 μg; ○—○, 1.0 μg; △—△, 0.1 μg; and □—□, no hapten control.

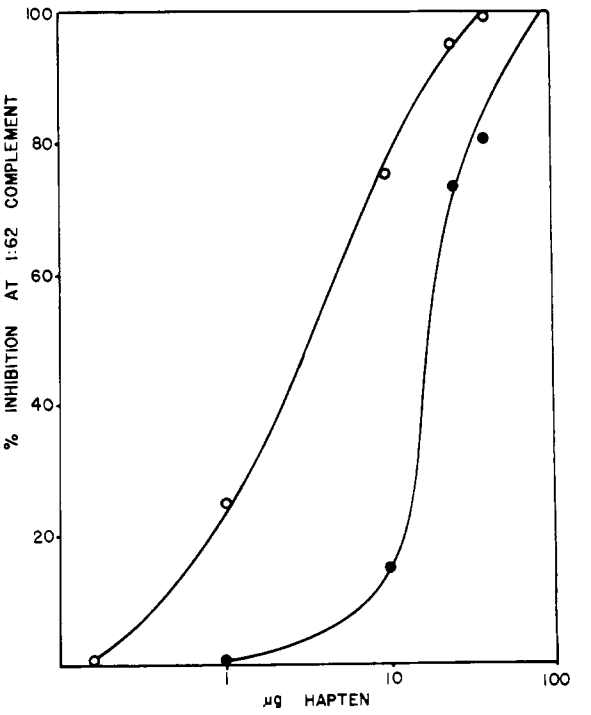

FIGURE 6: Correlation of inhibition of complement fixation at a 1:62 dilution of complement and varying concentrations of T-11 (○—○) and tryptic digest (●—●).

against native lysozyme did not cross react at the precipitation level with CM-lysozyme. The peptide (50 μg) was mixed with the antiserum and tested for its ability to inhibit the precipitation reaction as previously described. The peptide produced no detectable inhibition, indicating specificity for the CM-lysozyme system, and no affinity for the antiserum against the native system.

Discussion

The results presented here strongly indicate the participation of the peptide T-11 in the antigenicity of carboxymethylated lysozyme, by its ability to inhibit the homologous immunological reaction using both precipitation and complement fixation as test systems. The possibility that our inhibitory preparations of T-11 contained undigested protein or a contaminating peptide which could be responsible for the observed reactions was discounted on several grounds: the inability of the peptide to precipitate with antibody when tested either by ring test or by testing varying concentrations with antiserum, the amino acid analysis, which agrees closely with Canfield's (1963a) analysis of the peptide and appears to be virtually free of other amino acids, the very low levels at which this peptide demonstrated inhibition, and the lack of complement-fixing capacity on the part of the peptide preparations. The possibility that this effect was nonspecific was ruled out by the inability of T-11 to inhibit precipitation between native lysozyme and its homologous antiserum.

On a weight basis, T-11 was found to be approximately twice as efficient as the tryptic digest as an inhibitor, when tested either by precipitation or complement fixation. If this were the only determinant present in the digest, on a weight basis one would expect about five times the specific activity for the peptide since it comprises approximately 20% of the total molecule. This was never observed and at most three times the specific activity was noted. This does not discount the probability that other regions of the molecule possess antigenic properties, but rather that the T-11 region is the only one maintaining considerable activity after trypsin digestion. The specificity of this system does not appear to be related to individual animals since the sera from three different rabbits all reacted identically except with respect to antibody titer.

Peptide T-11 is the largest of the tryptic peptides found in CM-lysozyme, containing 23 residues occupying between the 74th and 96th position in the molecule. It has the sequences: NH_2-Asn-Leu-CMC-Asn-Ile-Pro-CMC-Ser-Ala-Leu-Leu-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CMC-Ala-Lys (Canfield, 1963b). It is interesting to note that this peptide bears similarities to the peptide T-8 from TMVP demonstrated by Benjamini *et al.* (1964) to have haptenic activity: it contains no aromatic amino acids, it is rich in nonpolar residues, and it is sparingly soluble in aqueous solutions. In further experiments on this peptide it is hoped that we will be able to determine more closely the sequence directly involved with antigenicity, as Benjamini's group (Young *et al.*, 1966) has done with the TMV peptide, T-8.

The most interesting aspect of these observations is that, although T-11 does not inhibit the precipitation reaction between native lysozyme and its homologous antiserum, the sequence Asn_{74} to Lys_{96} falls within the somewhat longer peptide Gln_{57} to Ala_{107} demonstrated by Shinka *et al.* (1967) to inhibit precipitation in the the native system. These workers found that the four peptic peptides they isolated all possessed the capacity to inhibit precipitation by 25% and all had closely similar amino acid compositions. They differed only with respect to their cleavage points within the loop between Cys_{80} and Cys_{94} which occurred at Leu_{83}, Leu_{84}, and Ser_{85}. These workers further digested their larger peptide with trypsin and found that one fragment was capable of delaying precipitation in the homologous system although they were unable to demonstrate over-all inhibition. This fragment, from observation of their amino acid analysis, appears to represent the sequences Asn_{74} to Ala_{96} and Trp_{62} to Arg_{68} joined at the Cys_{64} and Cys_{80} positions. From their observations, Shinka *et al.* (1967) concluded that this fragment represented an antigenic determinant. Their conclusions are in absolute agreement with our findings with CM-lysozyme although, with our system, inhibition of immunological reactivity by the T-11 peptide was far more marked. It is possible that the determinants may be the same for native lysozyme and the reduced and alkylated product even though the two antisera do not appear to cross react; and that radical differences in conformation are responsible for this. However, further work on these systems is necessary before this can be established.

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