Antibodies to a Continuous Region at Residues 38–54 of Hen Egg White Lysozyme Found in a Small Fraction of Anti-Hen Egg White Lysozyme Antibodies†

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ABSTRACT: The antigenic reactivity of the region at residues 38–54 of hen egg white lysozyme (lysozyme) was investigated by using various rabbit, goat, and sheep anti-lysozyme antisera by the charcoal–dextran method. This continuous antigenic region was completely independent in its specificity from other discontinuous antigenic regions of lysozyme. The antibodies to this region were shown to be reactive with native lysozyme as well as thermolytic nondisulfide peptides (residues 38–54 and 38–57) and synthetic peptides (sequences 38–48 and 34–54) by the following evidence. (1) The antigen binding activity could be inhibited by the addition of native lysozyme. (2) The antibodies to this region could be absorbed with lysozyme immunoadsorbent. (3) The specific antibodies prepared by the synthetic peptide (sequence 38–48) immunoadsorbent could neutralize the enzymic activity of lysozyme. (4) The synthetic peptide sequence 34–54 had the same inhibitory activity to the antigen binding as thermolytic nondisulfide peptide residues 38–54 and 38–57. The antibodies were found in both early (rabbit, at 6 weeks) and late (rabbit, goat, and sheep, from 1 month until 5 years) anti-lysozyme antisera. The amounts of these antibodies in various anti-lysozyme antisera, calculated from the binding capacity, were in the range from 11 to 70 μg per mL of sera. These values corresponded to a small fraction of the total precipitable anti-lysozyme antibodies and were as high as 1.0% of the total. The antibodies in part had relatively high avidities in comparison with those of antibodies to other discontinuous regions of lysozyme, and the range was 4.0×10^6 to 2.1×10^8 L per mol.

Several antigenic regions of native hen egg white lysozyme (lysozyme)[1] were reported previously. The first antigenic region is around a disulfide bond, Cys_6-Cys_{127} (Fujio et al., 1968a,b; Maron et al., 1971; Ha et al., 1975; Komatsu et al., 1975; Atassi et al., 1973, 1976a; Atassi & Lee, 1978). The second region is around a "loop" region containing two disulfide bonds, Cys_{64}-Cys_{80} and Cys_{76}-Cys_{94} (Shinka et al., 1967; Arnon & Sela, 1969; Maron et al., 1971; Teicher et al., 1973; Fujio et al., 1974; Atassi et al., 1973, 1975, 1976b,c; Lee & Atassi, 1975, 1977b; Lee et al., 1976). The third region is around a disulfide bond, Cys_{30}-Cys_{115} (Atassi et al., 1973; Lee & Atassi, 1977a). The presence of the disulfide bond is necessary for these three antigenic regions. The antigenic region of this type is named a "discontinuous" site (Atassi & Smith, 1978).

Recently, Atassi and co-workers have reported the precise and entire antigenic structure of native lysozyme (Atassi & Lee, 1978; Atassi, 1978). The antigenic regions and structures were investigated by using chemically modified lysozyme, peptides isolated from lysozyme, and "surface-simulation" synthetic peptides which were synthesized to simulate the surface amino acid sequence of the antigenic site. According to these authors, only three antigenic sites exist in lysozyme, and all of these sites are "discontinuous". However, evidence was reported from several other laboratories that additional antigenic regions might exist in a lysozyme molecule (Maron et al., 1972; Sakato et al., 1972; Arnon et al., 1974; Fainaru et al., 1974; Matthyssens et al., 1974; Fujio et al., 1974; Wilson & Prager, 1974; Ibrahimi et al., 1979).

In a previous communication from our laboratory (Sakato et al., 1972), the thermolytic disulfide peptide residue 29-54-(Cys_{30}-Cys_{115})-109-123 was found as one of the antigenic reactive peptides of lysozyme. Furthermore, it was found that thermolytic nondisulfide peptides residues 34–54 and 38–57 which consisted of a part of this disulfide peptide, had an inhibitory activity to the binding of [^{125}I]lysozyme with rabbit anti-peptide 29-54-(Cys_{30}-Cys_{115})-109-123 antibodies. These facts suggest the existence of the "continuous" antigenic region, which does not contain the disulfide bond.

We report here further evidence of the existence of antibodies to the continuous antigenic region at residues 38–54 of lysozyme in various rabbit, goat, and sheep anti-lysozyme antisera.

Experimental Procedures

Purification of Thermolytic Nondisulfide Peptides. Lysozyme (6× crystallized, Seikagaku Kogyo Co., Ltd.) was hydrolyzed with thermolysin (3× crystallized, Seikagaku Kogyo Co., Ltd.) at a substrate to enzyme weight ratio of 1000:1. To a stirred lysozyme solution (500 mL of 20 mg/mL in 50 mM Tris-HCl buffer containing 5 mM $CaCl_2$, pH 7.5) preincubated at 70 °C for 20 min, the thermolytic solution (10 mL of 1.0 mg/mL in the same buffer as above) was added and kept for 5 min. The reaction was stopped by adding 0.1 M Na_3-EDTA (30 mL), and the pH was adjusted to 3.5 with glacial acetic acid. The hydrolysate was roughly fractionated by SP-Sephadex C-25 chromatography. The acidic peptide fraction was chromatographed on Sephadex G-25 (fine) to remove the peptides of small size. The fraction containing P38–54 and P38–57 was further purified by QAE-Sephadex A-25 chromatography. Final products were desalted with gel filtration on Sephadex G-25 (fine) and lyophilized. Starting with 10 g of lysozyme, 304 mg of P38–54 and 249 mg of P38–57 were obtained. Amino acid sequences of these peptides are given in Figure 1.

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[1] Abbreviations used: lysozyme, hen egg white lysozyme; CM-lysozyme, reduced and S-carboxymethylated derivative of lysozyme; P38–54 and P38–57, thermolytic nondisulfide peptides of lysozyme residues 38–54 and 38–57, respectively; [^{14}C]acetyl P38–54, P38–54 acetylated at the α-amino group by [1-^{14}C]acetic anhydride; SP38–48 and SP34–54, synthetic peptides of lysozyme residues 38–48 and 34–54, respectively; Na_3-EDTA, trisodium ethylenediaminetetraacetate; BSA, bovine serum albumin; PBS, 0.01 M phosphate-buffered saline.

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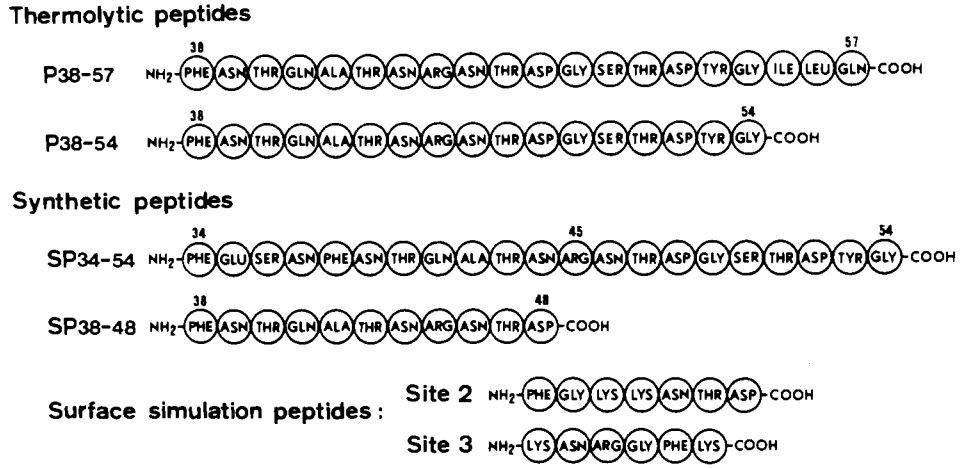

FIGURE 1: Amino acid sequences of thermolytic nondisulfide peptides of lysozyme and synthetic peptides. Structures shown are based on the proposed sequences of lysozyme by Canfield & Liu (1965) and of "surface-simulation" synthetic peptides by Lee & Atassi (1977a,b).

Characterization of P38–54 and P38–57. The amino-terminal analysis was performed by previously described methods on a polyamide sheet (Gray & Hartley, 1963; Woods & Wang, 1967). The carboxy-terminal analysis was carried out by hydrolysis with carboxypeptidase Y (Oriental Yeast Co., Ltd.) at a substrate to enzyme weight ratio of 200:1 at 35 °C, pH 5.5 (Hayashi et al., 1973, 1975). The released amino acids at various incubation times were quantitated by an amino acid analyzer. The homogeneity of P38–54 was checked with high-pressure liquid chromatography on a μBondapak/C18 column (7.8 mm × 30 cm) using a linear gradient from 0.01 M ammonium acetate, pH 4.0, to 50% methanol (v/v) in the same solution at a rate of 8.0 mL/min. Lysozyme contamination in P38–54 and P38–57 was also checked by measuring its lytic activity using *Micrococcus lysodeikticus* (Seikagaku Kogyo Co., Ltd.) as a substrate.

Preparation of Proteolytic Disulfide Peptides. The peptic disulfide peptide residues 1-27-(Cys_6-Cys_{127})-123-129 (Fujio et al., 1974; Ha et al., 1975), the tryptic disulfide peptide residues 62-68-(Cys_{64}-Cys_{80})-74-96-(Cys_{76}-Cys_{94}) (Fujio et al., 1974), and the thermolytic disulfide peptide residues 29-37-(Cys_{30}-Cys_{115})-109-123 (Sakato et al., 1972) were prepared according to previously described methods.

Synthesis of Peptides. The synthesis of protected peptides was carried out by the conventional stepwise elongation procedure using the active ester, starting with the amino acid benzyl ester. All amino acids except glycine were of the L configuration. The *tert*-butoxycarbonyl (Boc) group was used for the blocking of the α-amino group of each amino acid. The benzyl (Bzl) group was selected for protection of the ω-carbonyl group of aspartic acid and of the hydroxyl group of the threonine residue. The ε-amino group of lysine was blocked by the benzyloxycarbonyl (Z) group and guanidine, the side chain of arginine by the NO_2 group. The *N*-hydroxysuccinimide esters (Anderson et al., 1964; Danho & Li, 1971) were used, except for the *p*-nitrophenyl esters of Boc-asparagine, Boc-glutamine (Marshall & Merrifield, 1965), and Boc-(ε-Z)-lysine (Suzuki, 1966). For the coupling of the active ester, the Boc group was removed with 50% trifluoroacetic acid in methylene chloride at room temperature for 1 h. A 20% excess of esters was employed, in the presence of triethylamine in dimethylformamide, for the elongation of the peptide ester trifluoroacetate at 4 °C for 24 h. The completion of the coupling was monitored with thin-layer chromatography on a silica gel G-60 plate. The purification of intermediated protected peptides was performed by the usual methods. The final products were checked by melting point, thin-layer chromatography, and elemental analysis. Figure 2 summarizes the synthetic procedures of the protected peptides, 38–48, site 2, and site 3.

Purification of Synthetic Peptides. The final protected peptides (200 mg in 100 mL of 80% aqueous acid) were catalytically hydrogenated on 10% Pd/carbon (200 mg) at room temperature for 48 h. The catalyst was filtered off and the filtrate was lyophilized. The crude peptides were subjected to gel filtration on a Sephadex G-15 column (3 × 140 cm) equilibrated and eluted with 0.1 N acetic acid. The fraction containing the peptide was pooled and lyophilized. The yields of these peptides were about 90%.

Amino Acid Analysis. The amino acid compositions of proteolytic peptides and synthetic peptides were analyzed in a Hitachi KLA-5 analyzer after hydrolysis under reduced pressure in 6 N HCl at 110 °C for 24 h (Spackman et al., 1958).

Antisera. Rabbits were immunized by injecting the footpads with lysozyme (2.0 mg in 0.4 mL) in complete Freund's adjuvant. Booster injections were given 5 weeks after the first injection and once every 5 weeks thereafter with the same dose in incomplete Freund's adjuvant. Bleeding was performed 1 week after each booster injection. Goat and sheep were immunized with lysozyme (10 mg in 1.0 mL) in complete Freund's adjuvant at axillary and inguinal regions. Booster injections were the same as above. Antisera from the individual animals (goat and sheep) and pooled antisera (rabbit) were kept separate and stored frozen at −25 °C.

Preparation of Immunoadsorbents. The immunoadsorbents were prepared according to Pharmacia's manual. The active CH-Sepharose 4B (Pharmacia Fine Chemicals) was used for coupling the ligand. The contents of ligands were determined by amino acid analysis (6 N HCl, 110 °C, 24 h).

Preparation of the 7S Fraction of Sera. The 7S fractions of preimmune sera and antisera were prepared by 50% saturated ammonium sulfate fractionation and gel filtration on Sephadex G-200.

Preparation of Sheep Anti-SP38–48 Antibodies. Sheep anti-lysozyme antiserum (S60, 10 mL) was applied (6 mL/h) to a SP38–48 immunoadsorbent column (1.3 × 3 cm) at room temperature, and the column was washed with PBS, pH 7.2, until the absorbance of the effluent at 280 nm reached 0.01. Then the antibodies were eluted by a 6.0 M guanidine·HCl solution, pH 7.2, collected (containing 10 mL of 5% BSA), and dialyzed against PBS (pH 6.0, 5 L, twice). The final volume was adjusted to 50 mL with PBS, pH 6.0. Approximately 50% of the initial binding activity was obtained.

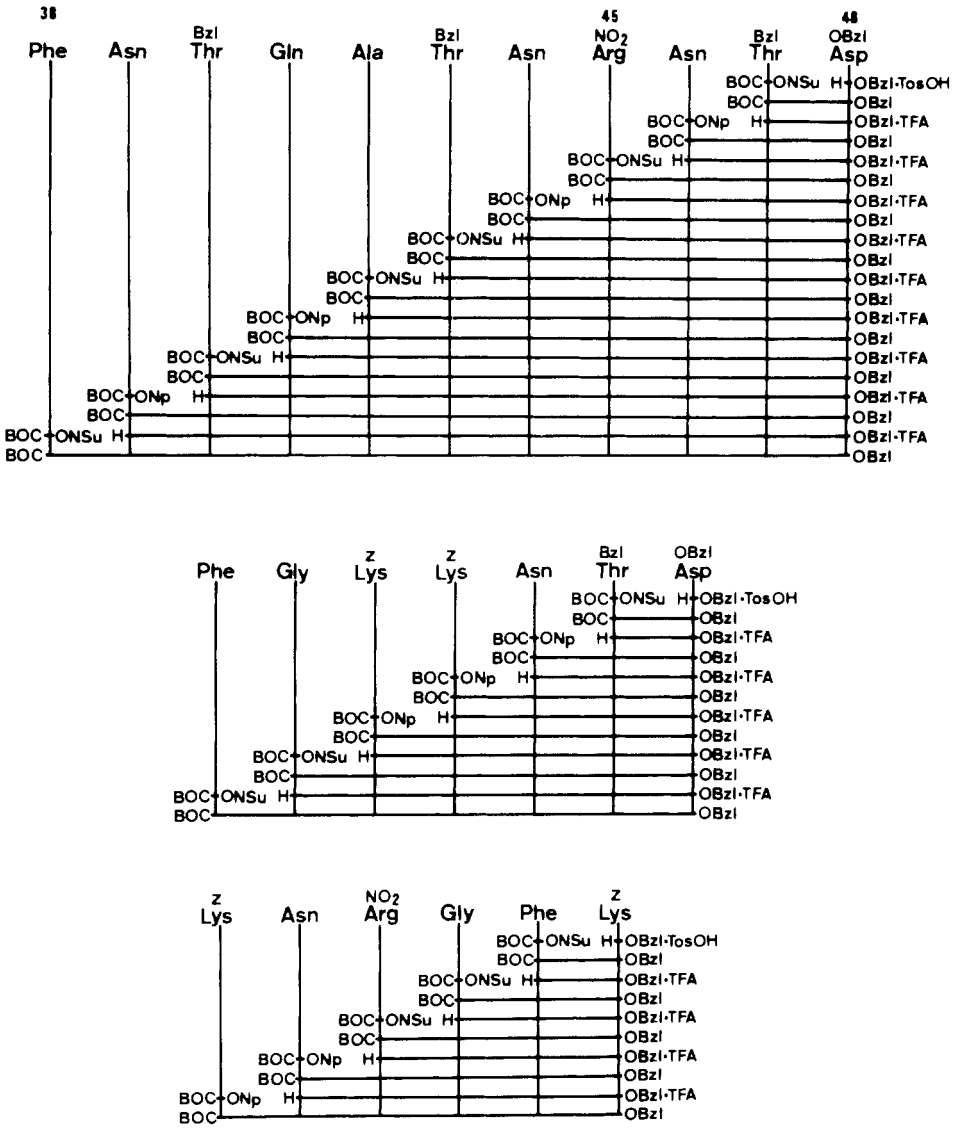

FIGURE 2: The synthetic procedures of protected peptides, residues 38–48 of lysozyme (top) and "surface-simulation" synthetic site 2 (middle) and site 3 (bottom): BOC, *tert*-butoxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; ONSu, *N*-hydroxysuccinimide ester; ONp, *p*-nitrophenyl ester; TosOH, *p*-toluenesulfonate; TFA, trifluoroacetic acid.

Neutralization of Enzymic Activity of Lysozyme by Anti-SP38–48 Antibodies. The antibody solution (1.0 mL, 0.025 nmol of antigen bound per mL in PBS, pH 6.0) was mixed with the lysozyme solution (0.1 mL, 2.0 μg/mL). After the solution was incubated for 15 min at 37 °C, a *Micrococcus lysodeikticus* suspension (1.0 mL, 0.5 mg/mL) was added and the solution was incubated at 37 °C for 30 min. The lytic activity of the enzyme was measured by the decrease of absorbance at 540 nm. The decrease of lytic activity with antibody is expressed as a percentage of that without antibody.

Labeling of P38–54 by [1-^{14}C]Acetic Anhydride. To a P38–54 solution (1.38 μmol in 2 mL of dimethyl sulfoxide) was added [1-^{14}C]acetic anhydride (4.04 μmol of 117.9 mCi/nmol in 0.5 mL of benzene, Radiochemical Centre, Amersham), and the solution was stirred for 2 h at room temperature. The excess reagent was removed by passage through a Sephadex G-15 column (3 × 24 cm) equilibrated with 1.0 N acetic acid. The fraction containing the peptide was pooled, lyophilized, and dissolved in PBS (pH 7.2, 20 mL). The specific activity of this solution was 41.2 nCi/mL.

Preparation of Charcoal–Dextran Mixture for Radioimmunoassay. The charcoal–dextran mixture was prepared according to a previously described method (Gottlieb et al., 1965) except that the veronal buffer was substituted with PBS, pH 7.2 (Curd et al., 1976). Equal volumes of the charcoal suspension (50 mg/mL) and dextran T70 (Pharmacia Fine Chemicals) solution (5 mg/mL) were mixed. This charcoal–dextran mixture can be stored up to 1 month at 4 °C.

Binding Assay. The 1:5 diluted antisera (0.4 mL), 1% BSA (0.1 mL), and the labeled antigen (0.5 mL) were mixed in a series of tubes. The mixture was incubated for 1 h at 0 °C. After the charcoal–dextran mixture (1.0 mL) was added to each tube, the mixture was centrifuged immediately (2000*g*, 10 min, 4 °C); the supernatant (1.0 mL) was mixed with scintillant (10 mL) and counted for 10 min in a Beckman Scintillation System LS-350. Each assay was performed in duplicate. The values of the antigen binding were corrected for nonspecific binding of 1:5 diluted preimmune sera and for the volume of the charcoal (0.08 mL). The effects of incubation time on the antigen–antibody interaction and of the volume of the charcoal–dextran mixture on the amount of antigen binding were tested. The results showed that 1 h was enough to reach an equilibrium, and the volume did not affect it within the range from 0.5 to 1.5 mL.

Inhibition of Antigen Binding with the 7S Fraction of Sheep Anti-Lysozyme Antiserum by Proteolytic and Synthetic

Table I: Amino Acid Composition[a] and Molecular Weight[b] of Synthetic Peptides

	synthetic peptides							
	SP34-54[c]		SP38-48		site 2		site 3	
amino acids	calcd	found[d]	calcd	found[d]	calcd	found[d]	calcd	found[d]
Asp	6	5.84	4	4.01	2	1.96	1	1.03
Thr	4	3.85	3	3.02	1	1.02		
Ser	2	1.81						
Glu	2	2.08	1	1.02				
Gly	2	1.92			1	0.98	1	1.02
Ala	1	1.00	1	1.04				
Tyr	1	0.87						
Phe	2	1.92	1	0.93	1	1.04	1	1.02
Lys					2	2.01	2	2.01
Arg	1	1.00	1	0.96			1	0.93
total residues	21		11		7		6	
mol wt	2339.3		1281.3		808.9		748.9	

[a] Results are expressed as residues per mole of peptide. [b] Calculated from the primary structures (shown in Figure 1). [c] A gift of Dr. Y. Shimonishi from the Institute for Protein Research, Osaka University. [d] Hydrolyzed for 24 h under reduced pressure in 6 N HCl at 110 °C.

Peptides. The peptide solutions (0.25 mL) having various concentrations were mixed with the 7S fraction (0.4 mL) and 1% BSA (0.1 mL) in a series of tubes. The reaction mixtures were incubated at 0 °C for 1 h. The labeled antigen (0.25 mL of 0.334 nmol/mL) was added to each tube. After further incubation under the same conditions, the charcoal–dextran mixture (1.0 mL) was added and centrifuged immediately. The supernatant (1.0 mL) was counted. The bound counts per minute (cpm) were corrected for nonspecific binding of the 7S fraction of preimmune sera. The decrease of cpm bound with inhibitor is expressed as a percentage of that without inhibitor. The percent inhibition of binding was plotted against the inhibitor per antigen molar ratio.

Results

Characterization of Synthetic Peptides. All synthetic peptides gave the expected molar ratio on amino acid analysis as shown in Table I. For good agreement in amino acid compositions, the synthetic peptides were used without further purification, except for gel filtration on Sephadex G-15. Amino acid sequences of the synthetic peptides are given in Figure 1.

Purification and Characterization of Thermolytic Nondisulfide Peptides. P38–54 and P38–57 were purified in four steps as shown in Figure 3. The amino acid compositions of these peptides are given in Table II. The amino- and carboxy-terminal residues of P38–54 were phenylalanine and glycine, respectively. Other amino acids were not detected. The homogeneity of P38–54 was checked by high-pressure liquid chromatography. A symmetric single peak was observed and its retention time was 18.2 min. Lysozyme in P38–54 and P38–57 was not detected from the measurement of its lytic activity, and the amount was under 0.01% (w/w).

Antigen Binding Activity of Serum Fractions Separated on Sephadex G-200. Sheep anti-lysozyme antiserum (S60, 3.0 mL) was applied to a Sephadex G-200 column (2 × 140 cm) equilibrated and eluted with PBS, pH 7.2. The binding activity of each fraction was measured. Of the total binding activity, 99% was found in the 7S fraction. More than 96% of the binding activity was lost by treating the 7S fraction with rabbit anti-IgG antisera. This fact indicates that the serum component responsible for the binding is the antibody of 7S class.

Independence of Continuous Antigenic Region. Several antigenic reactive peptides, known to have different specificities, which derived from different portions of lysozyme were tested for inhibitory activity to antigen binding with the 7S fraction of sheep anti-lysozyme antiserum (S60). In the case of three disulfide peptides, that is, 1-27-(Cys$_6$-Cys$_{127}$)-123-129, 62-68-(Cys$_{64}$-Cys$_{80}$)-74-96-(Cys$_{76}$-Cys$_{94}$), and 29-37-(Cys$_{30}$-Cys$_{115}$)-109-123, a 50 M excess of the labeled antigen was used. Synthetic sites 2 and 3 were tested by using a 200 M excess. All these peptides did not show any inhibitory activity (Table III). The results indicate that the antigenic specificity of the continuous region of lysozyme is completely independent from any other antigenic regions, and the antigen binding activity is not due to the contaminants of other antigenic reactive peptides.

Table II: Amino Acid Composition[a] and Molecular Weight[b] of Thermolytic Nondisulfide Peptides

	thermolytic nondisulfide peptides			
	P38-54		P38-57	
amino acids	calcd	found[c]	calcd	found[c]
Asp	5	5.03	5	5.01
Thr	4	4.00	4	3.82
Ser	1	1.00	1	1.03
Glu	1	1.02	2	2.09
Gly	2	2.01	2	2.00
Ala	1	1.01	1	1.03
Ile			1	0.99
Leu			1	1.00
Tyr	1	0.98	1	0.95
Phe	1	0.98	1	1.00
Arg	1	0.99	1	1.02
N-terminal[d]		Phe		
C-terminal[e]		Gly		
total residues	17		19	
mol wt	1861.8		2216.2	

[a] Results are expressed as residues per mole of peptide. [b] Calculated from the primary structures (shown in Figure 1). [c] Hydrolyzed for 24 h under reduced pressure in 6 N HCl at 110 °C. [d] Identified as the dansyl derivative. [e] Carboxypeptidase Y was used for hydrolysis.

Antigen Binding Activity in the Supernatant of the Precipitin Reaction of Sheep Anti-Lysozyme Antiserum. Sheep anti-lysozyme antiserum (S60, 1.0 mL) and lysozyme solution of various concentrations (0.1 mL) were mixed in conical tubes and incubated at 37 °C for 1 h and at 4 °C for 12 h. After incubation, tubes were centrifuged (2000*g*, 30 min). The binding activity of the 1:5 diluted supernatant with PBS, pH 7.2, was measured. The precipitate was washed 3 times with PBS (3.0 mL) and dissolved in 0.1 N NaOH (10 mL). The absorbance of each tube was measured at 280 nm. As shown in Figure 4, about 20% of the binding activity was inhibited at an equivalence point. However, when the amount of ly-

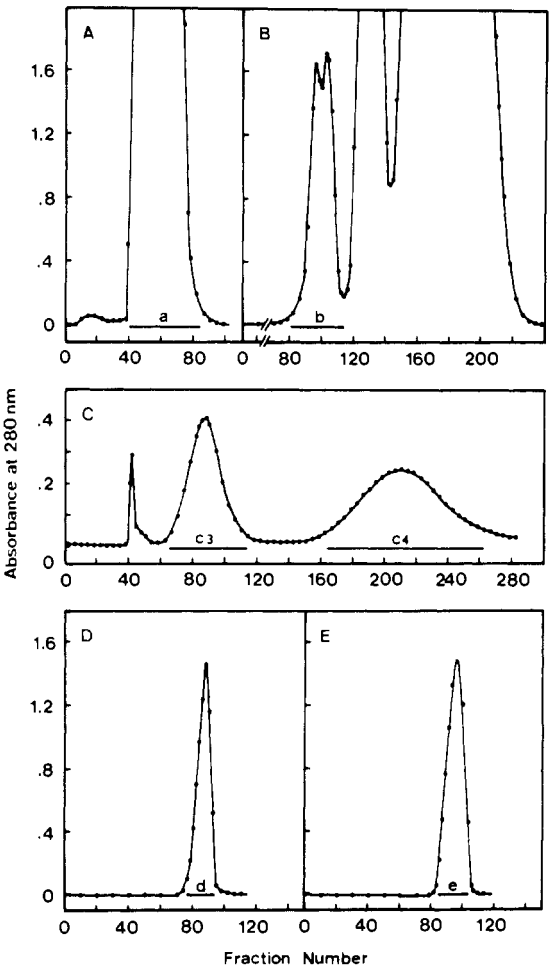

FIGURE 3: Purification of thermolytic nondisulfide peptides, P38–54 and P38–57. (A) Elution profile of thermolytic hydrolysates of lysozyme on a SP-Sephadex C-25 column (3 × 40 cm). Elution was performed with 0.16 M sodium acetate buffer (pH 4.5) at 200 mL/h and 20-g fractions were collected. Fraction a was lyophilized and dissolved in 50 mL of 0.1 N acetic acid. (B) Gel-filtration pattern of fraction a on a Sephadex G-25 (fine) column (4 × 140 cm). Elution was carried out with 0.1 N acetic acid at 100 mL/h and 10-g fractions were collected. Fraction b was lyophilized and dissolved in 20 mL of 0.03 M sodium acetate buffer (pH 6.0). (C) Elution profile of fraction b on a QAE-Sephadex A-25 column (2 × 40 cm). Elution was performed with 0.03 M sodium acetate buffer (pH 5.0) at 100 mL/h and 10-g fractions were collected. Fractions c3 and c4 were lyophilized separately and dissolved in 20 mL of 1.0 N acetic acid. (D) Gel-filtration pattern of fraction c3 on a Sephadex G-25 (fine) column (3 × 140 cm). Elution was carried out with 1.0 N acetic acid and 8-g fractions were collected. Fraction d was lyophilized. This peptide was identified as P38–57. (E) Gel-filtration pattern of fraction c4 on the same column and under the same conditions as in D. This peptide was identified as P38–54.

sozyme almost doubled, the binding activity was almost inhibited. The results indicate that the antibody populations capable of binding with P38–54 are also reactive to native lysozyme, and low affinity antibodies are present in these antibody populations. To avoid the reaction due to slight contamination, the homogeneity of lysozyme was checked by chromatographic analysis, electrophoretic analysis, ultracentrifugal analysis, N-terminal analysis, and amino acid composition. All of these data indicated that the lysozyme used in these experiments was highly pure.

Absorption of Anti-P38–54 Antibodies by Various Immunoadsorbents and Neutralization of Enzymic Activity of Lysozyme by Anti-SP38–48 Antibodies. Sheep anti-lysozyme antiserum (S60, 2.0 mL) was applied (6.0 mL/h) to the immunoadsorbent column (1.3 × 3 cm) at 4 °C, and the column

Table III: Inhibition of [^{14}C]Acetyl P38–54 Binding with the 7S Fraction of Sheep Anti-Lysozyme Antiserum (S60) by Other Antigenic Reactive Peptides of Lysozyme

inhibitors	molar ratio inhibitor/[^{14}C]Ac P38–54	[^{14}C]Ac P38–54 bound[a] (×10^{-11} mol) to 1.0 mL of 7S fraction	% inhibition
PBS (pH 7.2)		7.53	
62-68-(Cys_{64}-Cys_{80})-74-96-(Cys_{76}-Cys_{94})	50	7.51	0.3
1-27-(Cys_{6}-Cys_{127})-123-129	50	7.46	1.0
29-37-(Cys_{30}-Cys_{115})-109-123	50	7.49	0.5
synthetic site 2	200	7.32	2.8
synthetic site 3	200	7.50	0.5
P38–54	50	0.44	94.1

[a] Binding inhibition was performed by using 0.4 mL of the 7S fraction, 0.25 mL of the inhibitor (8.35 nmol/mL or 33.4 nmol/mL), 0.1 mL of 1% BSA, and 0.25 mL of [^{14}C]acetyl P38–54 (0.167 nmol/mL). The amount of [^{14}C]acetyl P38–54 bound was corrected for nonspecific binding by the sheep preimmune 7S fraction. Experimental details are given in the text.

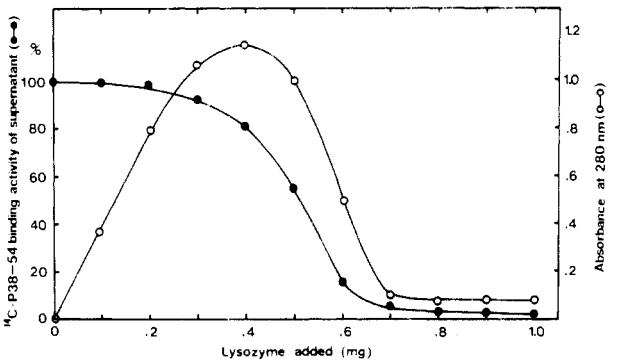

FIGURE 4: Precipitin reaction of sheep anti-lysozyme antiserum (S60) to lysozyme and [^{14}C]acetyl P38–54 binding activity of its supernatant. The binding assay was carried out at 0 °C for 1 h using 1:5 diluted supernatant (0.4 mL), 1% BSA (0.1 mL), and labeled antigen (0.5 mL of 0.334 nmol/mL). The relative binding activity was shown as a percentage to antigen bound of 1:5 diluted antiserum (0.4 mL) in the absence of lysozyme. Each point is the mean of two experiments: absorbance at 280 nm of the precipitate dissolved in 10 mL of 0.1 N NaOH (○); binding activity of supernatant (●). Experimental details are given in the text.

was washed with PBS, pH 7.2. The pass-through fraction was pooled (10 mL), and the antigen binding activity was measured by using the pass-through fraction (0.4 mL) and the labeled antigen (0.5 mL of 0.344 nmol/mL). As shown in Table IV, the lysozyme immunoadsorbent had the same ability to adsorb the antibodies to the continuous region as the P38–54 immunoadsorbent. However, the SP-38–48 immunoadsorbent had a slightly lower ability than these immunoadsorbents.

The neutralization of the enzymic activity of lysozyme was performed by using specifically purified anti-SP38–48 antibodies. About 89% of the lytic activity was neutralized by adding the antibodies at an antibody to lysozyme molar ratio of 1.7:1.

Antigen Binding Capacity and Avidity of Various Anti-Lysozyme Antisera. The calculation of avidity (K) is made with respect to total antibody sites (Abt) (Nisonoff & Pressman, 1958). The equation can be written as $1/b = (1/\text{Abt})(1/\text{Ag})(1/K) + 1/\text{Abt}$, where b is bound antigen and

Table IV: Absorption of Anti-P38-54 Antibodies in Sheep Anti-Lysozyme Antiserum (S60) by Various Immunoadsorbents

ligands	amt of ligand[a] (μmol/mL of wet gel)	[^{14}C]Ac P38-54 bound[b] to 1.0 mL of the pass-through fraction ($\times 10^{-10}$ mol)	% absorption
lysozyme	0.85	0.00359	98.2
P38-54	0.67	0.00348	98.3
SP38-48	0.38	0.0144	92.9
synthetic site 3	3.2	1.98	1.9
		2.03[c]	

[a] Determined by amino acid analysis, hydrolyzed under reduced pressure in 6 N HCl for 24 h at 110 °C. [b] Binding assay was carried out by using the pass-through fraction (0.4 mL), 1% BSA (0.1 mL), and the labeled antigen (0.5 mL of 0.334 nmol/mL). The values were corrected for nonspecific binding of 1:5 diluted sheep preimmune serum. [c] Antigen bound of the initial anti-lysozyme antiserum (1:5 diluted). Experimental details are given in the text.

Ag is free antigen. The value for Abt is obtained graphically by extrapolation of the binding curve to infinite free antigen concentration. The binding capacity is defined as the number of nanomoles of antigen bound by 1.0 mL of undiluted antisera under conditions of extreme antigen excess, and is calculated from the value for Abt. Anti-lysozyme antisera from 4 sheep, 3 goats, and 2 pools of 3 rabbits and 15 rabbits were tested for binding capacity and avidity. The binding curves and their double-reciprocal plots are given in Figure 5. The numerical values are shown in Table V.

Inhibitory Activity of Thermolytic Nondisulfide Peptides and Synthetic Peptides to Antigen Binding with the 7S Fraction of Sheep Anti-Lysozyme Antiserum (S60). As shown in Figure 6, the inhibition curves of thermolytic nondisulfide peptides, P38–54 and P38–57, could be superimposed with that of the synthetic peptide, SP34–54. These results indicate that the purity of thermolytic peptides, particularly with respect to contamination by other antigenic reactive peptides and native lysozyme, is as high as that of the synthetic peptide. More than 98% binding could be inhibited by these peptides. The inhibitory activity of SP38–48 was somewhat lower than that of other longer peptides. The control experiment was carried out by using the synthetic site 3 as inhibitor. The same results were obtained by using the 1:5 diluted sheep anti-lysozyme antiserum (S60) instead of the 7S fraction of antiserum.

Discussion

Atassi and Habeeb (1977; Atassi, 1978) pointed out the possibility that the partial proteolytic hydrolysis of lysozyme affords many intermediates even in finely purified peptides, and these peptides caused mistakes in the serious determination of the antigenic region. However, the thermolytic nondisulfide peptide, P38–54, was highly purified and characterized sufficiently. Furthermore, the antigenic purity of this peptide was ascertained by using the synthetic peptide corresponding to this region of lysozyme.

In our present work, we measured the direct binding of the thermolytic nondisulfide peptide with diluted antisera or the partially purified γ-globulin fraction to avoid the complications accompanying the use of purified antibodies, such as the selection of particular antibody populations and denaturation of antibodies by dissociating agents. The double-reciprocal plots of antigen binding with various anti-lysozyme antisera were derived from linearity and were bent at a certain point (Figure 5). These curves seemed to consist of two or three portions. These facts indicate the existence of several antibody populations having different affinities. In fact, avidities calculated from each point of the double-reciprocal plots had a wide range. For convenience, in Table V, avidities are expressed as 1/free antigen, where the binding reaches 50% of the maximum value. The heterogeneity of avidity, particularly the existence of low-affinity antibody populations, seems to explain the evidence shown in Figure 4. On the other hand, there are some antibody populations which have a relatively higher affinity than those of antibodies to other antigenic regions of lysozyme. However, avidities in early rabbit antisera (RP35) were about $^1/_{50}$ lower than those in late rabbit antisera (RP62). Increasing affinity during immunization is a general phenomenon, as found in the anti-hapten antibody system (Eisen & Siskind, 1964).

Judging from the evidence that antibodies to the continuous region at residues 38–54 can neutralize the enzymic activity of lysozyme, it is obvious that these antibodies can bind with native lysozyme. This fact was further ascertained by the evidence shown in Figure 4 and Table IV. To avoid the reactions due to slight contamination in lysozyme preparations, we have used highly purified and well-characterized lysozyme.

On the other hand, Young & Leung (1970) reported tryptic, chymotryptic, and cyanogen bromide cleavage products of reduced and S-carboxymethylated derivatives of lysozyme could not react with rabbit anti-lysozyme antisera. This fact indicates that antibodies to this continuous region do not exist in these anti-lysozyme antisera. However, we have also observed that these antibodies could not be found in guinea pig

Table V: [^{14}C]Acetyl P38-54 Binding Capacity and Avidity to Anti-Lysozyme Antisera in Different Species of Animals

antisera		bleeding at	binding capacity (nmol of antigen/mL of serum)	avidity[a] (L/mol)	anti-P38-54[b] antibodies (μg/mL of serum)	anti-lysozyme[c] antibodies (mg/mL of serum)
sheep	S10	3–5 years	0.83	1.1×10^8	62	6.2
	S20	3–4 years	0.39	1.1×10^8	29	4.5
	S60	1–16 months	0.93	1.1×10^8	70	6.8
	S70	1–12 months	0.16	3.3×10^7	12	11
rabbit	RP35	6 weeks	0.093	4.5×10^6	6.9	4.8
	RP62	16–31 weeks	0.15	2.1×10^8	11	3.3
goat	G1	6–12 months	0.60	6.5×10^7	45	9.3
	G2	6–12 months	0.29	6.0×10^7	22	4.5
	G20	6–12 months	0.42	4.0×10^6	31	4.5

[a] Avidity is expressed as 1/free antigen at which the binding reached 50% of the maximum value. [b] The numerical values are calculated from the binding capacity on the assumption that 2 mol of antigen can bind 1 mol of antibody. [c] Determined by the quantitative precipitin reaction. Experimental details are given in the text.

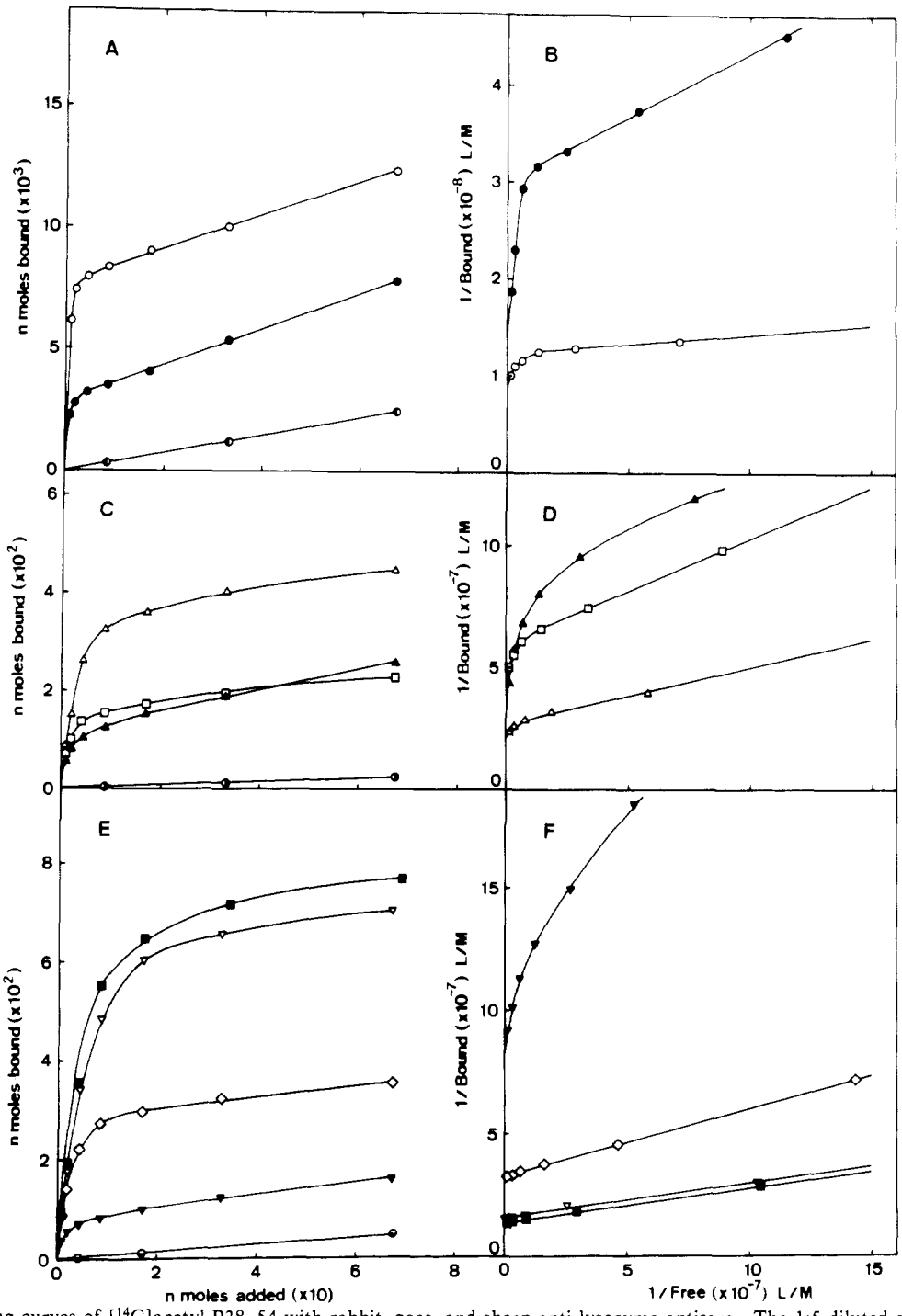

FIGURE 5: Binding curves of [^{14}C]acetyl P38–54 with rabbit, goat, and sheep anti-lysozyme antisera. The 1:5 diluted antiserum (0.4 mL) was tested against various concentrations of labeled antigen (0.25 mL), ranging from 0.206 to 1.32 nmol/mL. (A) Rabbit anti-lysozyme antisera: RP35 (●); RP62 (○); rabbit normal serum (◐). (B) Double-reciprocal plot of the data shown in A. (C) Goat anti-lysozyme antisera: G1 (Δ); G2 (□); G20 (▲); goat normal serum (◑). (D) Double-reciprocal plot of the data shown in C. (E) Sheep anti-lysozyme antisera: S10 (▽); S20 (◇); S60 (■); S70 (▼); sheep normal serum (◒). (F) Double-reciprocal plot of the data shown in E. All double-reciprocal plots were corrected for nonspecific binding of normal serum. Each point is the mean of two experiments. Binding capacity and avidity were calculated from graphs B, D, and F. Experimental details are given in the text.

(ST-13 and JY-1) and mouse (C3H/He) anti-lysozyme antisera. Therefore, the existence of these antibodies seems to depend on the species of animals or the individual animal.

Young and Leung also observed that antibodies from rabbits immunized with lysozyme were found to react with CM-lysozyme as well as lysozyme. All of the CM-lysozyme binding activity was retained in the anti-lysozyme antisera following absorption with lysozyme. The specificity of antibodies to CM-lysozyme was directed against the regions at residues 1–12 and 106–129. Scibienski (1973) also reported that antilysozyme antisera induced with the aid of complete Freund's adjuvant could be shown to react with the denatured form (CM-lysozyme), and the reaction of adjuvant-induced anti-lysozyme antisera with CM-lysozyme could not be inhibited by lysozyme. On the other hand, Gerwing & Thompson (1968) found that anti-CM-lysozyme antibodies reacted with only one tryptic peptide of CM-lysozyme representing residues 74–96. All of these observations indicate that antibodies to the denatured form of lysozyme cannot react with native lysozyme. On the contrary, antibodies to the continuous region

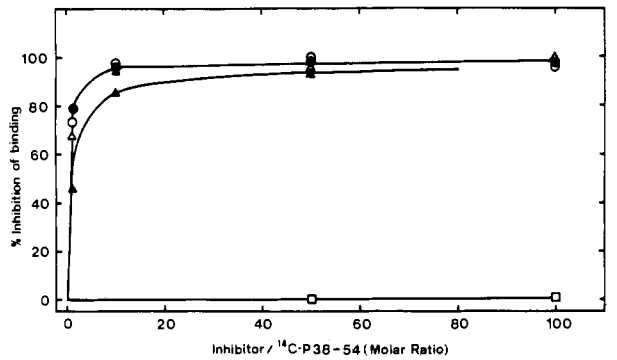

FIGURE 6: Inhibition of binding of [^{14}C]acetyl P38–54 with 7S fraction of sheep anti-lysozyme antiserum (S60) by thermolytic nondisulfide peptides and synthetic peptides. Binding inhibition was carried out by using the 7S fraction (0.4 mL), various concentrations of inhibitor (0.25 mL) ranging from 0 to 33.4 nmol/mL, 1% BSA (0.1 mL), and labeled antigen (0.25 mL of 0.334 nmol/mL). Each point is the mean of two experiments: P38–54 (Δ); P38–57 (●); SP34–54 (○); SP38–48 (▲); synthetic site 3 (□). Experimental details are given in the text.

at residues 38–54 could react with native lysozyme. Furthermore, this continuous antigenic region was different from the regions that antibodies to the denatured form of lysozyme could recognize.

Antibodies to the continuous antigenic region in various antilysozyme antisera are found in a small fraction of the total precipitable anti-lysozyme antibodies, and this level is as high as 1.0% of the total. However, knowing these antibodies exist is important in understanding the precise antigenic properties of lysozyme.

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