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The Identification of Aspartic Acid Residue 52 As Being Critical to Lysozyme Activity*

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ABSTRACT: A single ethyl ester derivative of lysozyme which retains inhibitor binding capability but which is catalytically inactive has been identified as occurring at aspartic acid residue 52 in the amino acid sequence.

Recent discussions of hen egg-white lysozyme generally have attributed its glycosidic activity to the side chains of Glu-35 and Asp-52. There is some kinetic evidence that carboxyls participate (Osawa, 1966; Raftery and Rand-Meir, 1968; Rupley and Gates, 1967). Also, modification of all "available" carboxyls inactivates the enzyme (Fraenkel-Conrat, 1950; Hoare and Koshland, 1967; Parsons *et al.*, 1969). However, the evidence that Glu-35 and Asp-52 are the specific carboxyls which are involved has been of a presumptive nature, based on the elegant results of Philips, Blake, and coworkers for the crystalline enzyme and on their model building (Blake *et al.*, 1967a,b, 1965; Blake, 1966).

We previously had utilized triethyloxonium fluoroborate to achieve mild selective esterification of carboxylates in lysozyme. We were able to isolate and partially characterize two single ethyl ester derivatives of the enzyme. One of these is enzymatically inactive but still retains the capability of binding the competitive inhibitor chitotriose. The details regarding the preparation and some of the properties of this inactive ester are given by Parsons *et al.* (1969). Figure 1 illustrates the chromatographic pattern obtained in the separation of a mixture of the lysozyme ester derivatives. Component II is the enzymatically inactive single ester discussed here. This communication describes the identification of this important carboxylic acid side chain.

Experimental Section

Materials. Hen egg-white lysozyme (lot 77B-8040) was purchased from Sigma Chemical Co. Bovine α-chymotrypsin (lot T-97207) was obtained from Armour Research Division, aminopeptidase M (lot 51132, 12,500 mEU/mg) from Rohm and Haas, subtilisin Carlsberg (lot 50624) from Novo Industries, Copenhagen, and CPA[1] (lot 762) from Worthington Biochemical Corp. Ninhydrin was a product of the Pierce Chemical Co. and aspartic acid was obtained from Eastman Kodak. Poly-L-glutamic acid was a product of Schwarz BioResearch, Inc. Standardization buffers for the pH meter were from Beckman.

The β-ethyl ester of aspartic acid was synthesized by refluxing a mixture of 5.0 g of DL-Asp-HCl and 1.1 g of anhydrous HCl in 50 ml of absolute ethanol for 15 min. The resulting warm solution was brought to cloudiness with dry ether and set aside (Bergmann and Zervas, 1933). Crystalline β-DL-ethylaspartic acid hydrochloride (2.8 g, mp 174.5–177.5°) was obtained. The structure was confirmed by nuclear magnetic resonance spectroscopy.

The γ-ethyl ester of glutamic acid was quickly synthesized in microamount by esterification of 1 mg of poly-L-glutamic acid with triethyloxonium fluoroborate in a manner similar to the lysozyme esterification. The esterified polymer was treated with subtilisin and aminopeptidase M as described below, yielding a solution containing essentially only L-glutamic acid and γ-ethyl-L-glutamate in about 1:3 ratio.

Analytical Methods

General Procedures. Acid hydrolysis of peptides was effected in constant-boiling HCl under vacuum for 20 hr at 105°. Base hydrolysis of protein samples was effected in 4 N barium hydroxide by the method of Noltman *et al.* (1962). Amino acid analyses were performed on a Beckman-Spinco Model 120B amino acid analyzer. The sodium buffer system usually employed was similar to that of Spackman (1963). Asparagine and β-ethylaspartic acid (β-EtAsp) were completely resolved from the other amino acids at 25° in a

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[1] Abbreviation used is: CPA, carboxypeptidase A.

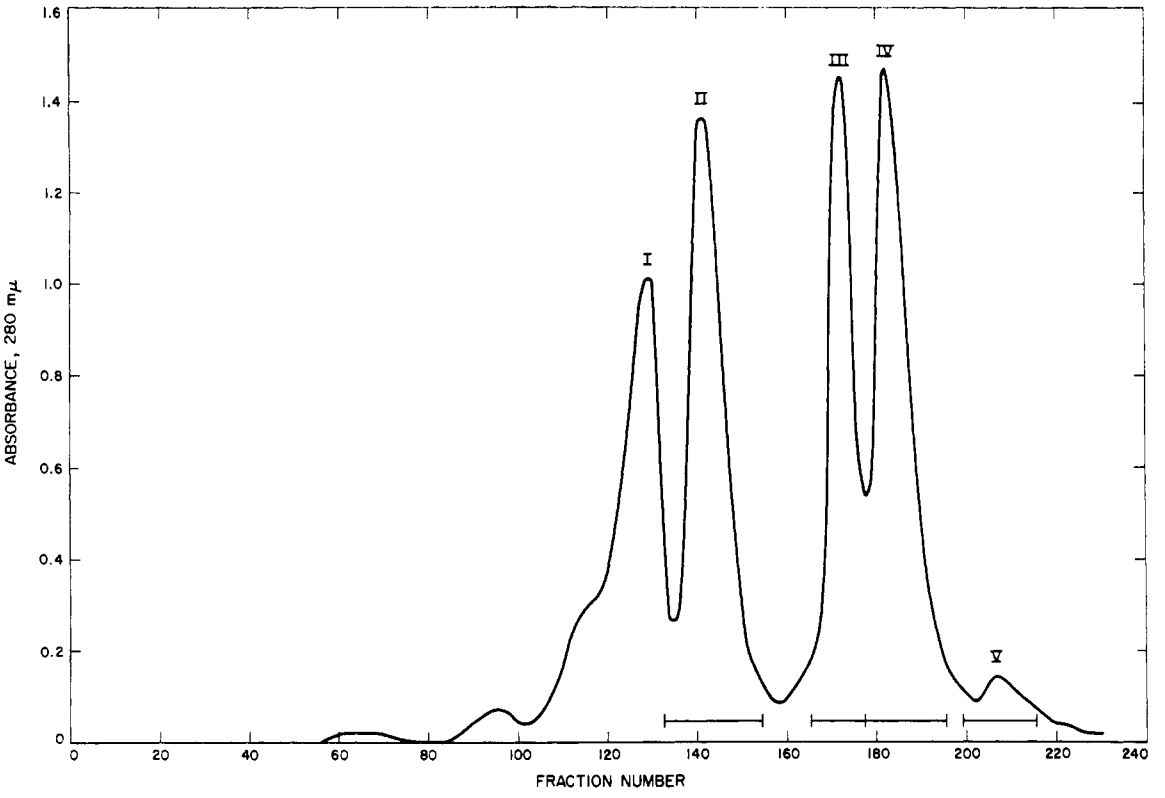

FIGURE 1: Preparative chromatography of ^{14}C-labeled lysozyme esters on Bio-Rex 70 (2.5 × 40 cm), 7°, pH 7.19, salt gradient from 0.16 M PO_4 (Na^+) (three chambers of 500 ml) to 0.20 M PO_4 (Na^+) containing 0.50 M KCl (one chamber of 500 ml), 8-ml fractions. Component I is native lysozyme; component II is an enzymatically inactive single ester derivative.

0.3 M lithium (citrate) buffer (pH 2.80), described in Technical Bulletin A-TB-044 from Spinco. Only the first buffer, eluting amino acids through glycine, was used. The values of amino acids normally found in acid hydrolysates were calibrated by standard mixtures (Beckman Co., Spinco Division). Other amino acids (β-EtAsp, Asn, Gln, and Trp) were calibrated with weighed amounts of the carefully dried amino acids.

Visible and ultraviolet absorbancies were determined with a Gilford Model 240 spectrophotometer. Scintillation counting of ^{14}C derivatives was performed in 15 ml of Bray's solution (Bray, 1960) in a Packard Model 527 scintillation spectrometer. Nuclear magnetic resonance spectra were obtained on a Varian Model A-60 spectrometer. A Radiometer PHM26 with a combination calomel–glass electrode was used to obtain pH readings.

Stability of the Inactive Ester. The ^{14}C-labeled inactive ester (2 mg) (see below for its isolation), also referred to as component II, which contained 1600 cpm was performic acid oxidized at −10° by the method of Hirs (1956). The product was taken up in 1.8 ml of water and a 0.8-ml aliquot was counted for radioactive content. To the rest, 0.1 ml of 0.2 M phosphate (pH 7.2) was added, the pH was adjusted to 7.3, and 0.1 mg of trypsin was added. After 9 hr at room temperature the protein was lyophilized and then taken up in 1.1 ml of H_2O. A 0.8-ml aliquot was counted. The original ester (0.6 mg) in 0.8 ml of water also was counted.

Total Enzymatic Digestion. A solution of 0.3 mg of subtilisin in 1.2 ml of 0.1 M phosphate (pH 7.0) was added to 5 mg each of oxidized native lysozyme and inactive ester and stirred at room temperature. After 3 hr the oxidized protein had completely dissolved. After 4 hr more, 2.0 mg of solid aminopeptidase M, which cleaves all peptide bonds (Pfleiderer and Celliers, 1963), was added. After 20 hr of further digestion, the solution was frozen. Aliquots were analyzed for amino acid composition in both the sodium and the lithium buffer systems.

An aliquot (0.20 ml) of each digest was treated with two drops of 1 N LiOH in 0.50 ml of water (to dilute the cation concentrations), giving a solution of pH 11. This was incubated at 40° for 30 min after which one drop of 6 N HCl was added and the solution was analyzed in the lithium buffer system.

α-Chymotryptic Digestion. Oxidized native lysozyme or inactive ester (5 mg) was dissolved in 1.5 ml of 10^{-3} M phosphate and 10^{-3} M $CaCl_2$ (pH 7.0) .The acidic protein lowered the pH of the solution to about 4 where it is soluble. The digestion was carried out in a 12 × 50 mm test tube fitted with magnetic stirring, a combination pH electrode, and a micrometer-mounted 1-ml syringe with No. 22 U-bent delivery needle. A solution of 0.3 mg of α-chymotrypsin in 0.3 ml of the same buffer was prepared in a separate syringe. The pH of the digest solution was rapidly raised to 7.0, precipitating the oxidized protein, and the α-chymotrypsin quickly injected. The pH was rapidly brought up to 6.98 ± 0.03 and maintained at that pH with 0.1 N NaOH. The mixture cleared in 10 min. After 4 hr at 25° the base in the syringe was changed

to 1 N HCl and the pH of the peptide solution lowered to 3.10. This solution was applied immediately to the peptide column.

Peptide Chromatography. Chromatography of peptide solutions was performed on columns (0.9 × 17 cm) maintained at 35.0° of a spherical, nominally 7.5% cross-linked, sulfonated polystyrene resin from Spinco, Type PA-35 (Hill and Delaney, 1967). The eluting buffer gradient was formed by means of a nine-chamber Autograd and was similar to one suggested by Schmidt (1966). Three stock buffers were prepared of (1) 0.2 N sodium formate adjusted to pH 3.20 with HCl, (2) 2.0 N sodium acetate adjusted to pH 5.10 with acetic acid, and (3) 2.0 N sodium acetate of about pH 6.8. Table I gives the initial compositions of the nine chambers. A flow rate of 50 ml/hr, which resulted in a pressure of 300 psi, was maintained by means of a Milton Roy Model 196 positive displacement pump. The entire effluent was fed into the amino acid analyzer. The analyzer ninhydrin system was operated in the usual manner and the resulting peptide map was recorded at slow chart speed (Hill and Delaney, 1967). A period of 13 hr was required for the chromatography. The resin was regenerated in the column with 0.2 N NaOH (30 min) followed by the starting pH 3.20 buffer (1 hr) with no increase in back-pressure.

TABLE I: Buffer Gradient for Peptide Column.

Chamber	Buffer No. 1, pH 3.20 (ml)	Buffer No. 2, pH 5.10 (ml)	Buffer No. 3, pH 6.8 (ml)	Water (ml)
1	90	0	0	0
2	90	0	0	0
3	90	0	0	0
4	90	0	0	0
5	55	15	0	20
6	5	45	0	38
7	0	87	0	0
8	0	65	22	0
9	0	0	87	0

Preparative Methods

^{14}C-Labeled Inactive Ester. A mixture of lysozyme ethyl esters obtained by reaction with ^{14}C-labeled triethyloxonium fluoroborate had been prepared and analyzed previously (Parsons *et al.*, 1969). After the addition of 50 mg each of nonradioactive component II and component IV to this mixture as carriers, ^{14}C-labeled component II was isolated by the method previously described (Figure 1). The other components appearing in Figure 1 were isolated at the same time also, but will not be discussed further here.

α-Chymotryptic Digestion and Peptide Chromatography. The preparative digestion was similar to the analytical one, except that 25 mg of oxidized lysozyme or inactive ester was dissolved in 7.0 ml of dilute phosphate buffer contained in a 10-ml beaker and 1.5 mg of α-chymotrypsin in 0.5 ml of the same buffer was added. Chromatography of the resulting peptides was performed as before. The column effluent was collected in 5-ml fractions, but only in the region of interest indicated by the analytical results spanning the fourth to the seventh hours of chromatography. The peptide regions were located by manual ninhydrin analysis on a 250-μl aliquot of each fraction. The four fractions containing the peptide of interest or the region into which (for lysozyme) or from which (for the ester) the peptide moved were pooled.

Removal of Sodium Ions and Peptide Contaminants due to Peak Tailing. A pooled fraction was evaporated to 1 ml on a rotary evaporator under vacuum at 35°. The viscous solution was transferred with washing to a 10-ml graduated cylinder, made up to 2.1 ml, and mixed with 0.7 ml of isopropyl alcohol. This solution was chromatographed on a column (2.5 × 40 cm) of superfine Sephadex G-25 (defined) equilibrated in 25% isopropyl alcohol–water (v/v) which was 0.036 M with respect to ammonium formate and adjusted to pH 4.6 with formic acid. Fractions of 5 ml were collected. The peptide peaks were located by ultraviolet absorptivity at 230 mμ. Each ultraviolet-absorbing fraction was pooled, the isopropyl alcohol was evaporated under vacuum at 35°, and the resulting 25 ml of water solution was lyophilized. The residue was taken up in 1.00 ml of water and frozen to give a stock solution of peptide. Amino acid analyses were performed both with and without acid hydrolysis on 0.10-ml portions of these stock solutions.

CPA Digestion of Peptide C-15. CPA (4 mg) was solubilized by the method of Fraenkel-Conrat *et al.* (1955) and made up to give a solution of 0.72 mg/ml of CPA in 0.2 M *N*-ethylmorpholine (acetate) at pH 7.6. This was treated with diisopropyl fluorophosphate (Potts, 1967). About 200 mμmoles of peptide C-15 (0.20 ml and 0.40 ml of the stock solutions from lysozyme and the ester, respectively) was made up to 1.00 ml with 0.10 ml of the CPA solution and 0.26 or 0.36 M morpholine buffer to give similar digest solutions containing 0.20 M *N*-ethylmorpholine (acetate) at pH 7.5. A blank lacking C-15 peptide was prepared also. These were incubated at 25° and 200-μl aliquots periodically were removed, pipetted into 0.5 ml of 0.2 M citrate (pH 2.2), and quick frozen until amino acid analysis. The 25-min sample from the ester digest was adjusted to pH 11 with NaOH, incubated at 35° for 1 hr, and then brought to pH 2.5 with HCl.

Results

The elution pattern obtained from the separation of the ^{14}C-labeled oxonium salt esterified derivatives appears in Figure 1. About 30 mg of the labeled inactive protein (Parsons *et al.*, 1969) (750 cpm/mg), component II, was obtained from fractions 133–154. When this labeled ester was oxidized in performic acid, 90% of the counts were recovered after lyophilization, indicating that the ester was stable to this treatment. Incubation of oxidized labeled component II at pH 7.2 for 9 hr at room temperature showed that 90% of the counts surviving oxidation were recovered after lyophilization, again indicating the stability of the ester group to this treatment.

Basic hydrolysis of the inactive protein indicated that its tryptophan content was identical with that of lysozyme. Acid hydrolysis previously had shown the same results for the other amino acids (Parsons *et al.*, 1969). The total enzy-

TABLE II: Amino Acid Compositions of Total Enzymatic Digests Determined in Sodium Buffer System.

Amino Acid	Amino Acid Ratios[a]	
	Lysozyme	Inactive Ester
$CySO_3H$	4.7	4.9
Asp	5.7	4.8
$MetSO_2$	1.4	1.2
Thr	Unresolved	
Ser + Asn + Gln	Unresolved	
Glu	1.8	1.9
β-EtAsp	0	0.97
Pro	1.7	1.7
Gly	9.2	9.0
Ala	11.7	12.0
Val	6.1	6.0
Ile	6.3	6.5
Leu	8.5	9.0
Tyr	3.2	3.4
Phe	3.2	3.1
His	0.96	0.92
Lys	6.1	6.2
Arg	8.2	8.0

[a] Residues per lysozyme molecule.

TABLE IV: Amino Acid Compositions of 5.5- and 6-hr α-Chymotryptic Peptides (Sodium Buffer).

Amino Acid	Amino Acid Hydrolyzed (mμmoles)	
	Lysozyme 5.5-hr Peptide	Inactive Ester 6-hr Peptide
$CySO_3H$	0	0
Asp	2880 (3)	1415 (3)
$Met\text{-}SO_2$	0	0
Thr	1934 (2)	960 (2)
Ser	910 (1)	446 (1)
Glu	0	Trace
Pro	0	Trace
Gly	1124 (1)	549 (1)
Ala	0	Trace
Val	0	Trace
Ile	0	Trace
Leu	0	Trace
Tyr	1054 (1)	530 (1)
Phe	0	Trace
His	0	0
Lys	0	Trace
Arg	1112 (1)	543 (1)

matic hydrolysis gave values for the amino acid composition in reasonable agreement with the accepted composition (Canfield, 1963; Jollès *et al.*, 1963), except for low values of aspartic acid, glycine, and arginine (see Tables II and III). The incomplete yield of aspartic acid always occurred and has been noted in other cases of total enzymatic digestion (Hill and Schmidt, 1962). It is apparent from Tables II and III that the ester derivative gave the same ratios of amino acids as native lysozyme except for one less Asp. One additional peak after Glu and partly resolved from Pro was observed in the amino acid analyzer trace of the ester digest run in the usual sodium system. This position corresponded to that of authentic β-ethylaspartic acid. No other new significant peaks occurred which were not present in the lysozyme digest. No peak at the position of γ-ethylglutamic acid, between valine and isoleucine, was observed. Since the proline peak has a low 570-mμ color intensity, the β-EtAsp peak could be integrated even though it was only half-resolved. The presence of β-EtAsp in the ester digest was further shown by the identity of the new peak with authentic β-EtAsp in a lithium buffer system, where it again occurred between Glu (plus Gln) and Pro but this time was completely resolved. The β-EtAsp peak is very distinctive since it possesses a greater color intensity in the 440-mμ channel than in the alternate 570-mμ channel. The 570-mμ color intensity given by β-EtAsp was reproducibly about one-third that of most other amino acids. Treatment of the ester digest with dilute base resulted in the disappearance of the peak attributed to β-EtAsp and in an increase equal to one residue of the aspartic acid peak (Table III).

In the proteolytic digestion with α-chymotrypsin most of the base uptake was complete after 2 hr. When the resulting peptide solution was chromatographed 18 peaks could be identified. The two analytical peptide maps obtained for lysozyme and the inactive ester were essentially identical except for a single major peak (Figure 2). The peak occurring at 5.5 hr in the chromatogram of the lysozyme digest had moved back to 6 hr in the ester pattern. A small peak due to ester-hydrolyzed peptide or a different peptide also falling at 5.5 hr remained in the position formerly occupied by the ester peptide. This provided evidence that the shift in position was significant.

TABLE III: Amino Acid Compositions of Total Enzymatic Digests Determined in Lithium Buffer System.

Amino Acid	Amino Acid Ratios[a]			
	Lysozyme	Inactive Ester	Lysozyme after pH 11	Inactive Ester after pH 11
$CySO_3H$	4.2	4.3	4.1	4.4
Asp	5.6	4.5	5.4	5.5
Thr	7.0	7.1	6.9	7.1
Ser	9.8	9.6	9.7	9.8
$Met\text{-}SO_2$	Unresolved			
Asn	13.0	13.2	12.6	13.0
Glu + Gln	Unresolved			
β-EtAsp		0.88		0.0
Pro	1.4	1.3	1.3	1.3
Gly	8.0	8.2	8.0	8.1

[a] Residues per lysozyme molecule.

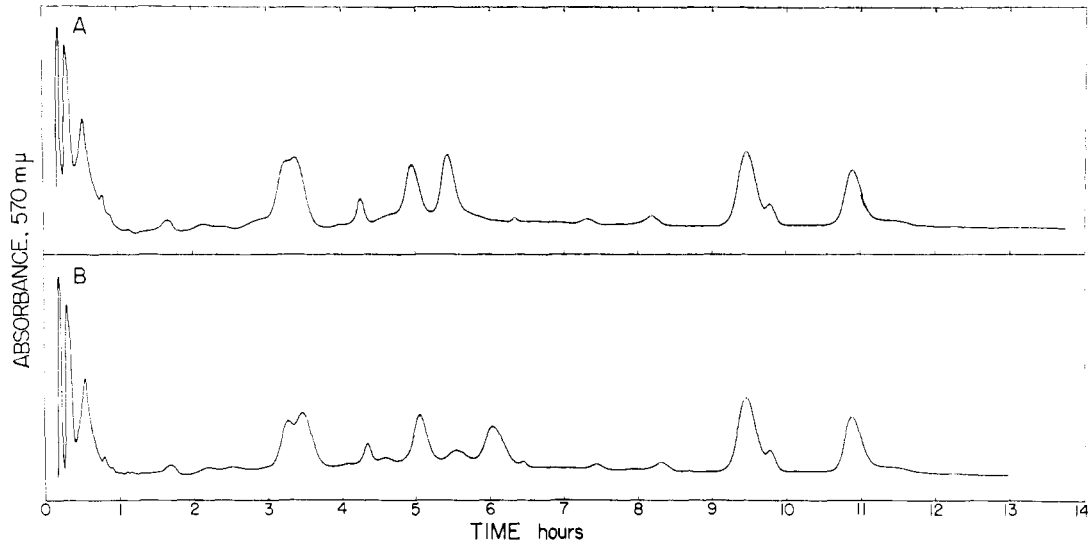

FIGURE 2: Analytical α-chymotryptic peptide map of performic acid oxidized proteins. Chromatography on Beckman-Spinco resin PA-35 (0.9 × 17 cm), 35°, nine-chamber gradient from 0.2 N sodium formate (pH 3.20) to 2 N sodium acetate (pH 6.8), amino acid analyzer ninhydrin system. (A) Lysozyme and (B) inactive ester. A single peptide peak in B moved from 5.5- to 6-hr elution time.

When the lysozyme peptide occurring at 5.5 hr, fractions 23–26 in Figure 3A, was isolated preparatively and chromatographed on Sephadex, three ultraviolet-absorbing peaks appeared which were preceded by an ill-defined forerun (see Figure 4A). The forerun was probably composed of contaminants due to peak tailing associated with peptide columns. Peaks 2 and 3 contained no amino acids after acid hydrolysis. Peak 3 was due to the concentrated sodium salts. Peak 1 gave the amino acid analysis after acid hydrolysis shown in Table IV. Without acid hydrolysis no amino acids were detected. The analysis corresponded well to peptide C-15 of Canfield's work which has the structure Arg-45, Asn-46, Thr-47, Asp-48, Gly-49, Ser-50, Thr-51, Asp-52, and Tyr-53 (Canfield and Anfinsen, 1963; Canfield and Liu, 1965). Two aspartic acid residues, at positions 48 and 52, occur in this sequence. The yield of peptide was 1000 mμmoles (60%).

Work-up in an identical manner of the region in the lysozyme pattern occurring at 6 hr, fractions 30–33 in Figure 3A, gave the Sephadex pattern shown in Figure 4A′. It was evident that no peptides of low ninhydrin reactivity occurred in this region.

When the new peptide from the inactive ester, fractions 30–33 in Figure 3B, was isolated preparatively, the Sephadex pattern shown in Figure 4B resulted. Amino acid analysis of the acid-hydrolyzed peptide revealed that it too was C-15 (Table IV). The yield was 500 mμmoles (30%). The yield, which is based on the weight of material oxidized, is low because of salt in the original isolated protein. No free amino acids occurred with the isolated peptide. Fractions 24–27 of Figure 3B gave the Sephadex pattern of Figure 4B′. This result indicated that little of the ester had hydrolyzed and that no peptide of low ninhydrin reactivity was present at 5.5 hr along with C-15.

CPA digestion of C-15 from lysozyme gave an immediate quantitative release of tyrosine-53 and a very slow release of aspartic-52 (Figure 5A), as expected (Ambler, 1967). No other amino acids were observed. The digestion of peptide C-15 from the ester gave quite different results. Tyrosine-53 was again quickly released. No aspartic acid was released but instead β-ethylaspartic acid was released at a moderate rate (Figure 5B). The increased rate of release of an uncharged β derivative of aspartic acid is expected (Ambler, 1967). Furthermore, a small amount of threonine-51 was released. No other amino acids were observed in significant amounts. When the 25-min sample from the ester peptide was treated with dilute base before amino acid analysis, 32.4 mμmoles of aspartic acid was found. This datum, plotted as

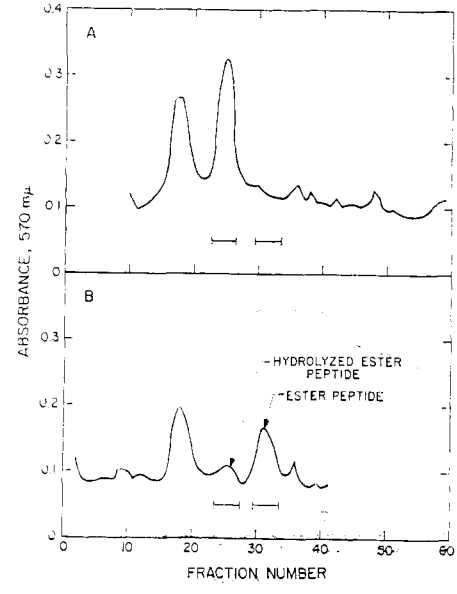

FIGURE 3: Preparative α-chymotryptic peptide chromatogram from the fourth to the seventh hours. Same chromatographic conditions as in Figure 2, 5-ml fractions, manual ninhydrin analyses. (A) Lysozyme and (B) inactive ester. Fractions enclosed by bars were pooled.

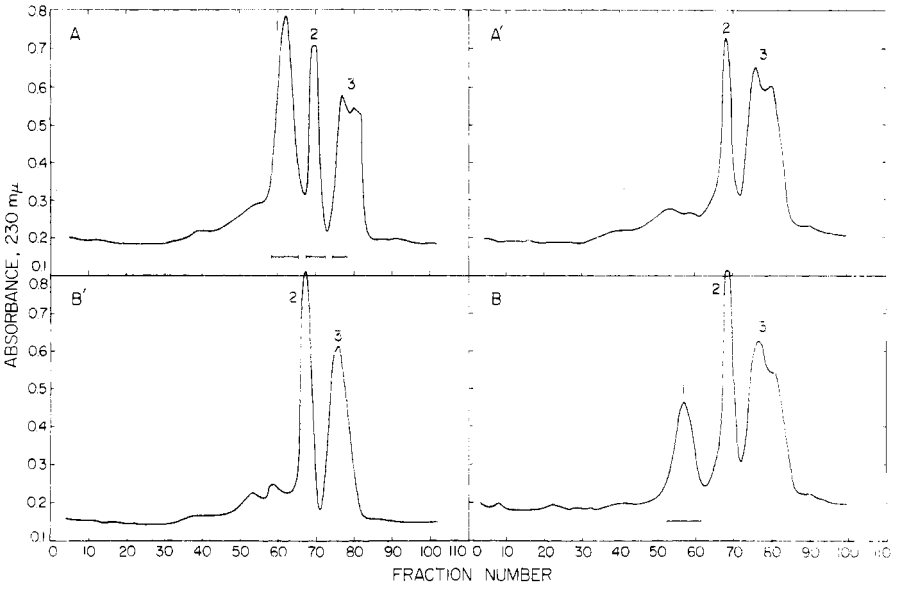

FIGURE 4: Sephadex G-25 chromatography of pooled regions from Figure 3. Chromatography in 25% isopropyl alcohol–0.036 M ammonium formate (pH 4.6), 5-ml fractions, 230-mμ absorptivity of each fraction read. (A) Lysozyme C-15, fractions 23–26 of Figure 3A; (A′) lysozyme, fractions 30–33 of Figure 3A; (B) inactive ester C-15, fractions 30–33 of Figure 3B; and (B′) inactive ester, fractions 24–27 of Figure 3B. Fractions enclosed by bars were pooled and analyzed for amino acid content.

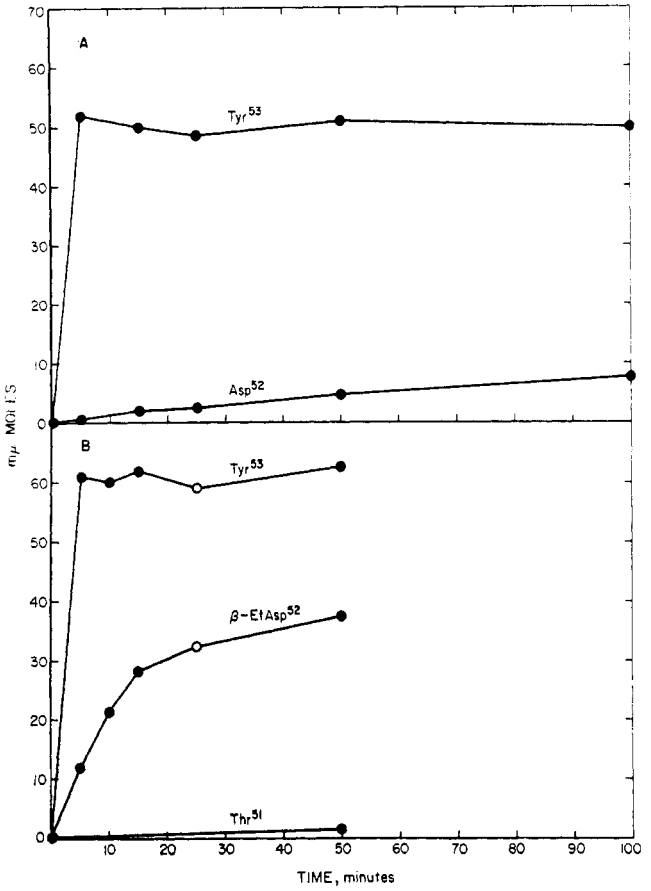

FIGURE 5: Release of amino acids from C-15 peptide by CPA in *N*-ethylmorpholine (pH 7.5) at 25°, *vs.* time. (A) Lysozyme and (B) inactive ester. Ester sample taken at 25 min (—○—) was treated with base before amino acid analysis. Other samples (—●—) were chromatographed directly.

mμmoles of β-ethylaspartic acid, fits into the curve of Figure 5B well. This is over ten times the amount of aspartic acid released from the native lysozyme peptide after 25 min.

Discussion

Before discussing the procedure which identified the inactive ester, we should mention briefly some early unsuccessful procedures. In order to avoid the possibility of ester hydrolysis, attempts were made to convert the inactive lysozyme derivative to a hydrazide or to a hydroxamic acid. Such ester conversions normally occur readily, can be made specific, and have been utilized previously on protein esters with success (*e.g.*, Blumenfeld and Gallop, 1962; Gallop *et al.*, 1959). However, with component II, none of the expected product could be detected although model esters and the component III ester underwent the expected conversions. $LiBH_4$ reduction (Wilcox, 1967) was tried, also without success. Even after vigorous treatment, resulting in the appearance of one-half residue of homoserine from native lysozyme, no additional homoserine was found from component II. Perhaps related to these properties is the fact that incubation of the inactive ester at pH 10 did not regenerate enzymatic activity, while incubation at pH 2 did (Parsons *et al.*, 1969). It is interesting to note that each of the above reactions which failed depends upon an initial nucleophilic attack on the ester while the one reaction which succeeded depends upon an initial protonation of the ester.

Component II was previously shown to be an ester by indirect means. The coupling of glycinamide to the oxidized protein by means of a carbodiimide (Hoare and Koshland, 1967) demonstrated the disappearance of one carboxyl group. Because component II failed to undergo typical ester reactions, further confirmation of its identity was desirable. The first step was to rule out the modification of the other amino

acids. Acid hydrolysis combined with basic hydrolysis completed the elimination of all the amino acids except Asp, Glu, Asn, Gln, and the C-terminal Leu as to having been ethylated by the oxonium salt. Assuming that the presence of an ester would be confirmed, the carbodiimide result rules out Asn and Gln. Also, the C-terminal sequence is known to be not critical (Jollès, 1967). After the radioactive label was shown to be stable to performic acid oxidation (also expected from the carbodiimide results) we sought to observe the ester directly. This was done by total enzymatic hydrolysis. The identification of one residue of β-ethylaspartic acid in the component II total enzymatic hydrolyzate was quite certain by virtue of three identical characteristics with authentic β-EtAsp in two different amino acid analyzer buffer systems. Because of its low specific radioactivity, the ^{14}C-labeled derivative was not utilized to locate the site of esterification. The α-chymotryptic peptide map readily showed that Asp-48 or Asp-52, occurring in C-15, was the site of esterification. The C-15 peptide is ideally suited for investigation of Asp-52 with carboxypeptidase A since the C-terminal Tyr-53 residue would be expected to separate rapidly and quantitatively, giving rise to an "internal standard," and to give no complications in the release of the second residue.

CPA digestion showed much more rapid release of β-EtAsp from the component II peptide than of Asp from the native lysozyme peptide. Because β-EtAsp-52 was observed directly, it was felt unnecessary to examine Asp-48 for modification since a negative result for an ester, which is hydrolyzable, would be inconclusive. The site of esterification in component II was therefore shown to be at Asp-52.

It was previously shown that this derivative is enzymatically inactive while retaining the capability of binding the inhibitor chitotriose. Thus, the above identification constitutes direct chemical evidence that the catalytic functionality of hen egg-white lysozyme occurs *in the region* of Asp-52. This result is in agreement with the hydrolytic mechanism suggested by Phillips and Blake (Blake, 1966; Blake *et al.*, 1967a). It is not, by itself, certain proof that Asp-52 and Glu-35 are indeed the participating groups. In a study complementary to this one, Lin and Koshland (1969) prepared an active lysozyme derivative in which all of the carboxyl groups except Asp-52 and Glu-35 were modified to an extent greater than the loss of enzymatic activity. The two studies together strongly indicate that Asp-52 and Glu-35 are in fact the important carboxyls.

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