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Osaka University

Regeneration
to the Native Form of Hen Egg-White Lysozyme
from its Protected Derivatives

Saburo Aimoto

1977

Regeneration to the Native Form of Hen Egg-White Lysozyme
from its Protected Derivatives

A Doctoral Thesis

Submitted by

Saburo Aimoto

Faculty of Science

Osaka University

1977

Dedicated to my parents

Acknowledgements

The work of this thesis was carried out at Institute for Protein Research, Osaka University from 1973 to 1977.

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INTRODUCTION

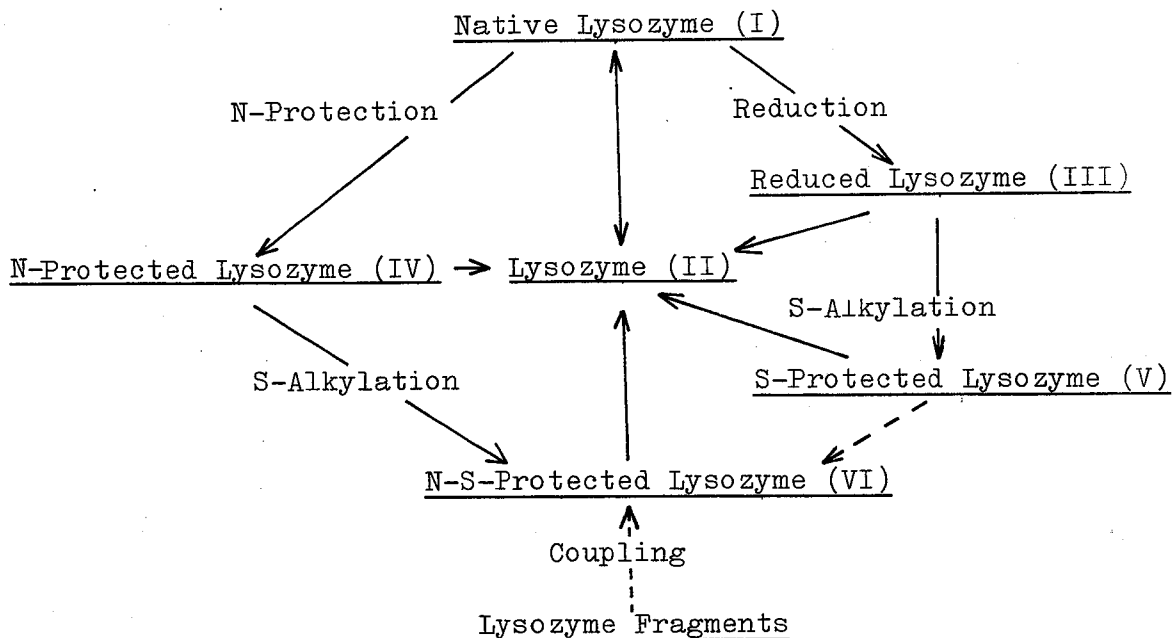
The chemistry of peptide has so far advanced that physiologically active peptides and peptide antibiotics can be easily synthesized in a pure form. A new target in peptide chemistry has been the total synthesis of proteins such as enzymes and hormones. Recently, Gutte and Merrifield¹⁾ reported the synthesis of bovine pancreatic ribonuclease A by the solid phase method,²⁾ which one of them had developed. However, they could not isolate the fully active enzyme. Furthermore, the investigation has been continued to synthesize various enzymes by conventional method in many laboratories in the past ten years, whereas no success has been achieved so far. The chemical synthesis of proteins will be accomplished in the near future judging from the great advancement of peptide chemistry. However, it seems very difficult to obtain desired products by means of either the conventional method or the solid phase method of peptide chemistry, because these methods have not taken into account the chemical and biological instabilities of biologically active polypeptides. Therefore, it is necessary to develop synthetic procedures suitable for proteins on the basis of not only peptide chemistry, but also protein chemistry.

The studies on the chemical synthesis of hen egg-white lysozyme have been carried out in our laboratory in the past

several years. Hen egg-white lysozyme is one of the enzymes of which biochemical and physicochemical properties have been studied extensively in many laboratories. Recently, Sharp et al.³⁾ reported the synthesis of hen egg-white lysozyme with 3-2% of the activity of the native hen egg-white lysozyme by the solid phase method. However, they could not purify the material further. The chemical synthesis of hen egg-white lysozyme seems very difficult, because the enzyme contains a high content of tryptophan residues which are subject to serious modification on chemical treatments and also the enzyme possesses labile asparagine residues which are easily deaminated under mild conditions. Therefore, it is necessary to improve present synthetic methods and to develop novel synthetic procedure for hen egg-white lysozyme, which should also be generally applicable to the synthesis of other proteins. This is the purpose of this thesis.

The chemical synthesis of proteins will be carried out by the following procedures; 1) synthesis of protected peptide fragments, 2) synthesis of protected polypeptide which covers the entire sequence of protein molecule by the assembly of protected peptide fragments, 3) removal of all the protecting groups from protected polypeptide, and 4) finally, folding the deprotected polypeptide to the native form of protein. In this thesis the procedures, 3) and 4), were mainly studied, related to the chemical synthesis of hen egg-white lysozyme, because the procedure 3) has never been established in any

protein and the chemical synthesis of proteins is impossible without the establishment of the procedure 3). The investigations were conducted as follows: as shown in Figure, first,



the influence of deprotecting reagents on native hen egg-white lysozyme (Route I → II), second, the influence of the deprotecting reagent, which was best in the deprotecting reagents examined, on the reduced hen egg-white lysozyme (Route I → III → II) and finally, the preparation of protected lysozyme derivatives, which are similar to protected polypeptides covering the entire sequence of the hen egg-white lysozyme molecule,

and the regeneration to the original enzyme from them (Route I \rightarrow IV \rightarrow II, I \rightarrow V \rightarrow II and I \rightarrow IV \rightarrow VI \rightarrow II).

Part I in this thesis deals with the problems concerning the influence of anhydrous liquid hydrogen fluoride on the native hen egg-white lysozyme. Many reagents have been used for removal of all the protecting groups from the protected peptides in the final step of peptide synthesis. However, some of them are known to cause serious side-reactions such as the cleavage of peptide bonds and the modification of the side chains of some kinds of amino acid residues. For example, sodium in liquid ammonia is known to cleave the peptide bond of imino site of the proline residue, and especially, to do very easily the threonylprolyl bond,⁴⁾ which exists in the hen egg-white lysozyme molecule. Moreover, hydrogen bromide in trifluoroacetic acid should not be used as a deprotecting reagent for protected peptides containing tryptophan residues, because both hydrogen bromide and trifluoroacetic acid degrade the indole nucleus of tryptophyl residues. On the other hand, anhydrous liquid hydrogen fluoride has never been known to bring about serious side-reactions, although the reagent causes N \rightarrow O acyl migrations of peptide bonds in peptides containing β -oxyamino acids, which can be rearranged reversibly to the normal state. Recently, deprotecting reagents such as methanesulfonic acid have been successfully developed by Yajima et al.⁵⁾ However, these reagents seem not to be extremely recommended in comparison with hydrogen fluoride.

Anhydrous liquid hydrogen fluoride was originally used in studies on the chemistry of peptides and proteins by Katz,⁶⁾ and Katz et al.^{6,7)} examined its effect on some natural polypeptide hormones, such as insulin and corticotropin, and also on enzymes such as ribonuclease and lysozyme. They reported that considerable inactivation of these compounds on exposure to hydrogen fluoride at a higher temperature (0 - 25°C) and for a longer time (≥ 2 h), particularly in the case of ribonuclease and lysozyme, while the biological activities of these compounds were not irreversibly affected by hydrogen fluoride, provided that the temperature of exposure was low (-78°C) and the exposure time was short (<2 h). These findings indicate that biologically active polypeptides may be considerably affected by prolonged exposure to hydrogen fluoride at high temperature. Thereafter, anhydrous liquid hydrogen fluoride was applied to peptide synthesis by Sakakibara et al.⁸⁾ and since then it has been widely used for releasing free peptides from the protected peptides, for the reason as mentioned above. However, in the synthesis of complicated biologically active polypeptides such as proteins, it is uncertain whether it can be used to release free polypeptides from protected derivatives without causing side-reactions. Therefore, the author intended to use anhydrous liquid hydrogen fluoride in this investigation.

Part II deals with the problems concerning the influence of anhydrous liquid hydrogen fluoride on the reduced form of

hen egg-white lysozyme. The native enzyme has a highly oriented structure. Therefore, its denaturation on exposure to hydrogen fluoride is considered to involve various factors such as destruction of three dimensional structure of the native enzyme, chemical modification of main and/or side chains in the native enzyme and so on. Furthermore, the influence of hydrogen fluoride toward the inner and outer part of the native enzyme are also assumed to be different. Therefore, it is necessary to investigate the effect of hydrogen fluoride on the reduced form of hen egg-white lysozyme, which can be prepared by unfolding of the native enzyme and has a random structure, because the aim of this thesis is in the findings of the conditions, under which protected derivatives of the reduced hen egg-white lysozyme should lead to a fully active enzyme.

In the course of investigations of the influence of hydrogen fluoride on the reduced form of hen egg-white lysozyme, the effect of various compounds, which were added into the solution of the reduced enzyme in hydrogen fluoride was examined. It was found that some of them bring about an increase of the recovery of the native lysozyme isolated from the hydrogen fluoride-treated reduced lysozyme.

Studies were also made on the effects of amino acid derivatives which liberate cations during the treatment with hydrogen fluoride, on the reduced hen egg-white lysozyme. Anisole has generally been used as a scavenger of cations liberated from protected amino acids and peptides in acidic medium⁹⁾ or

during the treatment with hydrogen fluoride.⁸⁾ Therefore, we also examined the effect of anisole during the treatment with hydrogen fluoride of the reduced hen egg-white lysozyme as preliminary experiments on treating the protected derivatives of hen egg-white lysozyme with hydrogen fluoride, whose preparation will be described in part III.

Part III deals with studies using the derivatives of hen egg-white lysozyme prepared from the native enzyme. The results in part II that the fully active original lysozyme can be recovered in a fair yield from the reduced hen egg-white lysozyme by the treatment with hydrogen fluoride in the presence of some thiol compounds suggest that the derivatives of the reduced hen egg-white lysozyme will lead to the native enzyme. In this case, the reduced hen egg-white lysozyme must be substituted with such protecting groups that can be removed by the treatment with hydrogen fluoride within short period at 0°C. In order to make this suggestion clear, the author attempted to prepare the derivatives of hen egg-white lysozyme substituted with various protecting groups from the native enzyme and to regenerate the original active enzyme from them by treatment with hydrogen fluoride.

Amino protecting groups were introduced onto the amino groups in the native hen egg-white lysozyme using various reagents. The protecting groups introduced were as follows; 1) t-butoxycarbonyl group which is readily removed from oligopeptides with formic acid, trifluoroacetic acid and hydrogen

fluoride, 2) benzyloxycarbonyl group which is gradually cleaved from peptides with reagents used for the removal of α -amino-protecting groups during peptide synthesis, when the protecting group is used as a protecting group for ϵ -amino group of lysine residue, and 3) benzyloxycarbonyl groups chlorinated in the aromatic ring which are more stable in acidic media than benzyloxycarbonyl group, and which are, therefore, recommended to be used as protecting groups for the ϵ -amino group of the lysine residue for diminishing the production of branching peptides. However, these chlorobenzyloxycarbonyl groups have only been examined to a limited extent.

For the purpose of protecting the thiol groups of cysteine residue, there are widely used three types of protecting groups which can be removed from peptides with sodium in liquid ammonia, or mercurous salt, or hydrogen fluoride. The treatment of the reduced form of hen egg-white lysozyme with either sodium in liquid ammonia or mercurous salt resulted in very low recovery of the original enzyme from the reduced hen egg-white lysozyme thus treated. Therefore, protecting groups which can be cleaved from peptides with hydrogen fluoride was investigated in detail. S-protecting groups such as benzyl, 4-methoxybenzyl and 4-methylbenzyl were introduced to thiol groups in the reduced hen egg-white lysozyme according to the method of Meienhofer et al.¹⁰⁾

Finally, the native hen egg-white lysozyme was acylated with amino protecting reagents, reduced with dithiothreitol

in liquid ammonia, and alkylated with S-protecting reagents. The derivatives of hen egg-white lysozyme thus prepared were treated with hydrogen fluoride. The results showed that 2-chlorobenzoyloxycarbonyl and 4-methylbenzyl substituents are extremely suitable for protecting amino and thiol groups, respectively, and that a fully active enzyme can be regenerated from the derivatives of hen egg-white lysozyme that are blocked with these protecting groups.

The results described in this thesis indicate clearly that the protected polypeptides covering the whole sequence of protein molecules lead to proteins. The strategy has been inferred from the success of the chemical synthesis of various biologically active peptides, but has never been realized on any protein. Now, the strategy has been established in the present investigation that the protected derivatives of hen egg-white lysozyme gives the fully active enzyme in a crystalline state after the treatment with hydrogen fluoride. The author hopes that the procedures described in this thesis will be extended to the total synthesis of hen egg-white lysozyme and also will be generally applicable to other proteins.

References

- 1) B.Gutte and R.B.Merrifield, J.Biol.Chem., 246, 1922 (1971).
- 2) R.B.Merrifield, J.Amer.Chem.Soc., 85, 2149 (1963).

- 3) J.J.Sharp, A.B.Robinson, and M.D.Kamen, J.Amer.Chem.Soc., 95, 6097 (1973).
- 4) A.Marglin and R.B.Merrifield, J.Amer.Chem.Soc., 88, 5051 (1966).
- 5) H.Yajima, H.Ogawa, H.Watanabe, N.Fujii, M.Kurobe, and S. Miyamoto, Chem.Pharm.Bull.(Tokyo), 23, 371 (1975).
- 6) J.J.Katz, Arch.Biochem.Biophys., 51, 293 (1954).
- 7) A.L.Koch, W.A.Lamont, and J.J.Katz, Arch.Biochem.Biophys., 63, 106 (1956).
- 8) S.Sakakibara and Y.Shimonishi, Bull.Chem.Soc.Jpn., 38, 1412 (1965).
- 9) S.Sakakibara, Y.Shimonishi, Y.Kishida, M.Okada, and H. Sugihara, Bull.Chem.Soc.Jpn., 40, 2164 (1967).
- 10) J.Meienhofer, J.Czombos, and H.Maeda, J.Amer.Chem.Soc., 93, 3080 (1971).

Part I

Influence of Anhydrous Hydrogen Fluoride on Native Hen Egg-White Lysozyme

In part I, the influences of anhydrous hydrogen fluoride (HF) on the native form of hen egg-white lysozyme (HEL) were investigated for establishing how much of the intact structure of this enzyme is retained after exposure to HF under usual conditions for peptide synthesis and how much of the original enzyme can be recovered in an intact form after this treatment.

Materials and Methods

Substrates and Reagents Spray-dried cells of Micrococcus Lysodeikticus (Lot. No. 21-88-778) were purchased from Seikagaku Fine Biochemicals. Sephadex was purchased from Pharmacia Co. (Uppsala) and hydrogen fluoride was a product of Daikin Ind. Co. (Osaka). Urea of reagent grade was recrystallized from ethanol before use. All other chemicals used were of analytical grade.

Hen Egg-White Lysozyme (HEL) Hen egg-white lysozyme (6 x crystallized Lot. No. 7103) from Seikagaku Fine Biochemicals was used without further purifications. It was dissolved in dilute acetic acid, lyophilized and then dried over phosphorus pentoxide in vacuo. The freeze-dried powder was used in all experiments.

Treatments of Native HEL with HF

The freeze-dried powder (ca. 30 mg) of native HEL was weighed in a Daiflon cylinder of the HF-reaction apparatus.¹⁾ HF (5 ml) was distilled into a cylinder containing HEL previously cooled to -78°C in a dry-ice/methanol bath. The reaction was started transferring the sample from the bath at -78°C to that at 0°C or 25°C , and then resulting solution was kept for the required period with stirring. After a given period, HF was completely removed from the reaction mixture by evaporation in vacuo, and the residue of the HF-solution of native HEL was dissolved in M/15 sodium phosphate buffer solution at pH 6.2 (30 ml), and stirred at room temperature for 20-24 hr. Then, its enzymatic activity was assayed.

Enzyme Assays

The specific activity of HEL after treatment with HF was determined by measuring the initial rate of lysis of Micrococcus lysodeikticus cells suspended in M/15 sodium phosphate buffer solution at pH 6.2 containing 0.1% sodium chloride at 37°C using the method of Jolles,²⁾ with six times recrystallized native HEL as a standard. The extent of lysis was measured spectrophotometrically at 540 nm.

Desalting of Protein

Protein solutions containing various salts were charged on a column of Sephadex G-10 (3 x 60 cm), and eluted with 0.1M acetic acid. The fractions of eluate with absorption at 280 nm were collected and lyophilized.

Gel-filtration

The lyophilized powder of the material

recovered after treatment of native HEL with HF was dissolved in a buffer containing 1M acetic acid and 5M urea and charged on a column of Sephadex G-50 (fine, 2.2 x 95 cm), equilibrated with the same buffer. The column was eluted with the same buffer and the absorption of each fraction at 280 nm was determined.

Ion-Exchange Chromatography The lyophilized powder obtained after treatment of native HEL with HF was dissolved in a small volume of 0.2M sodium phosphate buffer solution at pH 7.15 and charged on a column of Bio-rex 70 (1.9 x 54 cm), equilibrated with the same buffer. The absorptions at 280 nm of the fractions eluted with the same buffer were measured and fractions in each peak were combined and lyophilized.

Ultra-violet and Circular Dichroism Absorption Spectra

Ultra-violet absorption spectra were measured using a Hitachi UV-spectrophotometer type-124, equipped with a recording attachment. Circular dichroism spectra were measured in a Jasco automatic spectropolarimeter J-20, equipped with a CD-attachment. $[\theta]$ represents the molecular ellipticity based on the mean residue weight. Protein concentration was calculated using the value, $E_{1\text{cm}}^{1\%} = 26.9$ at 280 nm. The cell length was 1 mm for the range of wavelengths of 220 to 250 nm and 1 cm for those of 290 to 300 nm. The solvent was 0.1M sodium chloride, adjusted to pH 3.0 with hydrochloric acid. Spectra were measured at 25 °C.

Crystallization of HF-Treated HEL Crystallization was carried out by the method of Berthou and Jolles³⁾ with some modifications. The lyophilized protein (35 mg) was dissolved in distilled water (0.5 ml), and then first 0.2M acetate buffer solution at pH 4.7 (0.063 ml) and distilled water (0.187 ml) and then 10% sodium chloride solution (0.75 ml) were added. The protein concentration was adjusted to 2.33% and the solution was allowed to stand in a refrigerator at 5°C for 3 days. Crystals were deposited from solutions of enzymatically fully active materials.

Results

Treatment of Native HEL with HF The freeze-dried native HEL was first treated with HF at 0°C or 25°C. After a given period for 90 to 270 min, the reaction was stopped by complete evaporation of the HF from the HF-solution of the freeze-dried native HEL. The residue was dissolved in M/15 sodium phosphate buffer solution at pH 6.2 containing 0.1% sodium chloride and after 20-24 hr its lytic activity on Micrococcus lysodeikticus was compared with that of standard HEL. The enzymatic activity of the solution of the freeze-dried native HEL treated with HF at 0°C for 90 min was about 80% of that of standard HEL and decreased gradually on longer treatment, On the other hand, after treatment at 25°C for 90 min its activity was only 10% of that of standard HEL and after 270 min-treatment it was

almost inactive. These results were summarized in Fig. 1.

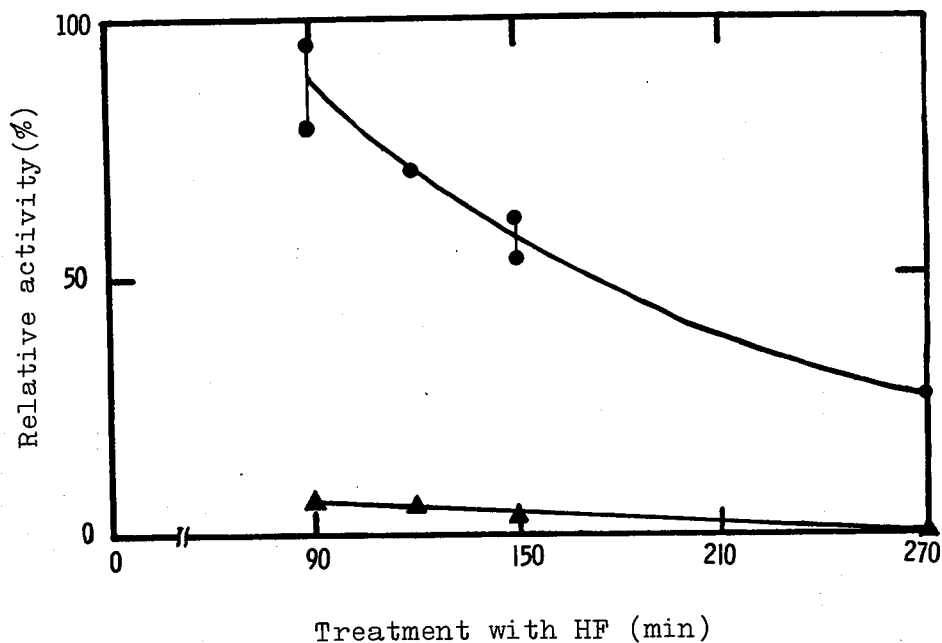


Fig. 1. Relative activity of HF-treated native HEL. The procedure used for treatment of HEL with HF and the method used of enzymatic assay of HF-treated HEL are given in the section of Materials and Methods.

●—● : treated at 50°C,

▲—▲ : treated at 25°C

Gel-Filtration of HF-Treated Native HEL on Sephadex G-50.

The material recovered from an HF-solution of the freeze-dried

native HEL was desalted on Sephadex G-10 and subjected to chromatography on Sephadex G-50 using buffer solution containing 1M acetic acid and 5M urea. The chromatogram of the material recovered after treatment of the freeze-dried native HEL with HF at 0°C for 90 min is shown in Fig. 2.

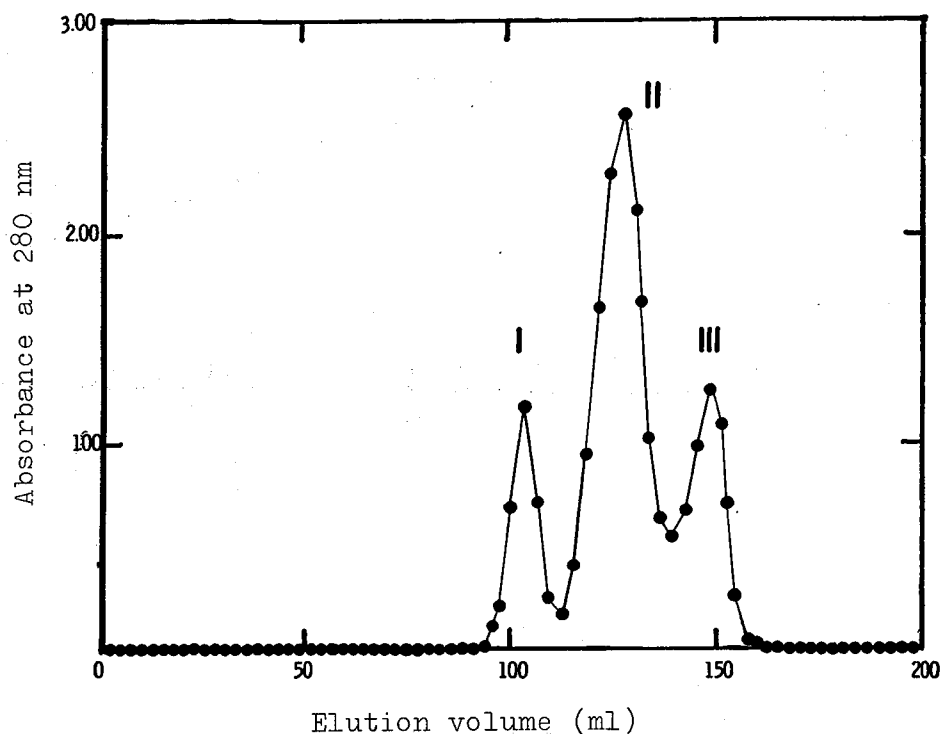


Fig. 2. Gel-filtration of native HEL treated with HF at 0°C for 90 min on Sephadex G-50 (2.2 x 95cm) using buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

Three fractions (I, II and III) were separated. The first fraction (I) was eluted in the void volume, the second fraction (II) before standard native HEL and the third fraction (III) in the same position as the native HEL under the same chromatographic conditions. Fraction II was subjected to Sephadex G-50 column chromatography using 5% NaCl and 5M urea buffer solution, and could be further separated into two fractions, IIa and IIb, as shown in Fig. 3.

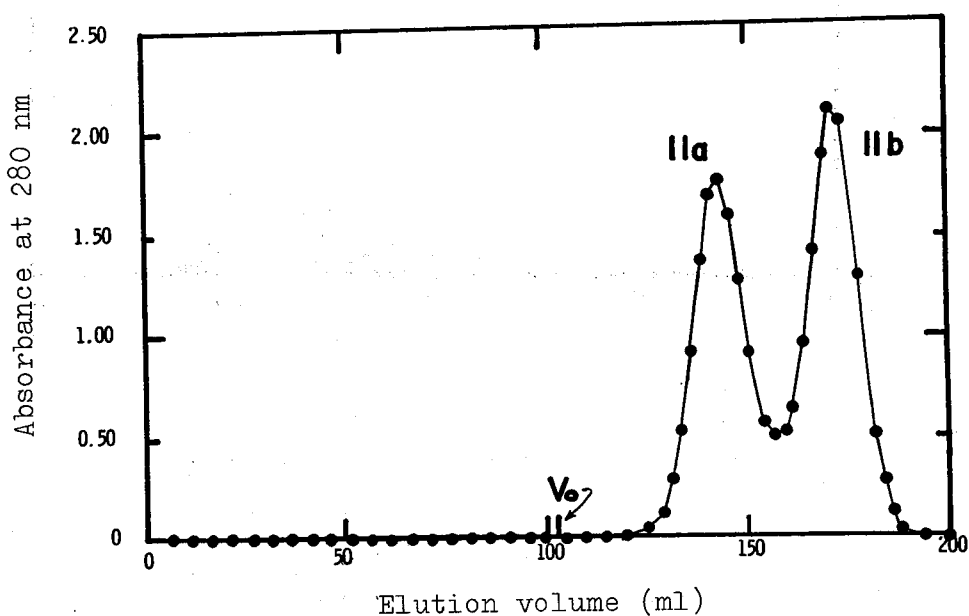


Fig. 3. Gel-filtration of fraction II of Fig.2 on Sephadex G-50 (2.2 x 95 cm) using buffer containing 5% NaCl and 5M urea. The flow rate was 10 ml per hr.

Fraction III was rechromatographed on Sephadex G-50 using buffer containing 1M acetic acid and 5M urea as shown in Fig. 4.

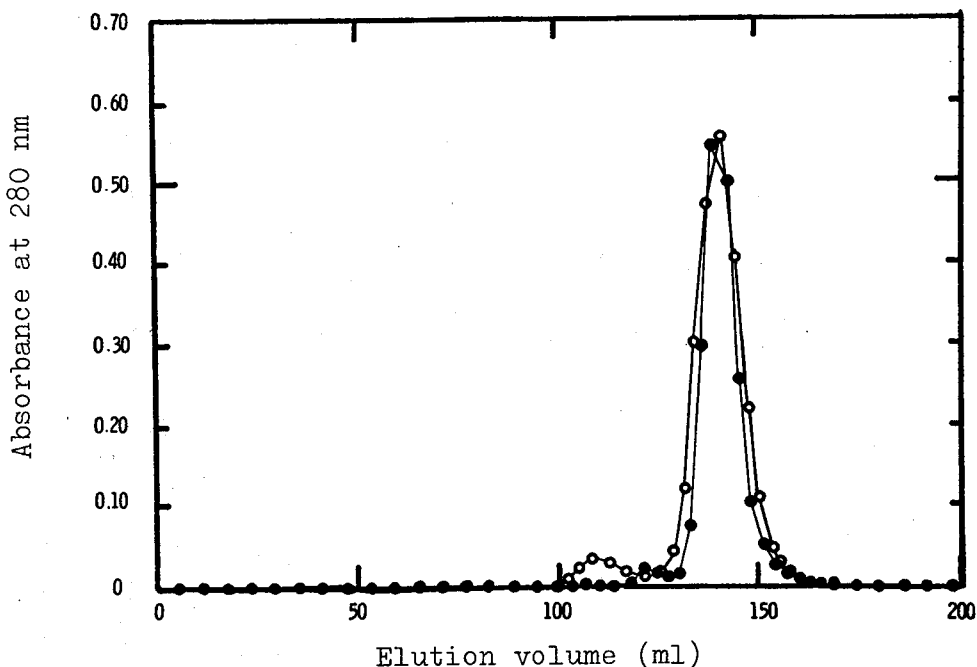


Fig. 4. Rechromatogram of fraction III of Fig. 2 on Sephadex G-50 (2.2 x 95 cm) using buffer containing 1M acetic acid and 5M urea.

The flow rate was 19 ml per hr.

- : fraction III of Fig. 2
- : native HEL

When the freeze-dried native HEL which had been treated with HF under various conditions was subjected to gel-filtration on Sephadex G-50 using buffer containing 1M acetic acid and 5M urea, different chromatograms were obtained depending on conditions of HF-treatment. On prolongation of the reaction

period at a given temperature the amount of fraction II increased and the amount of fraction III decreased. Furthermore, it was found that with a constant reaction period the amount of fraction III decreased on increasing the reaction temperature. Typical chromatograms are shown in Figs. 5 and 6.

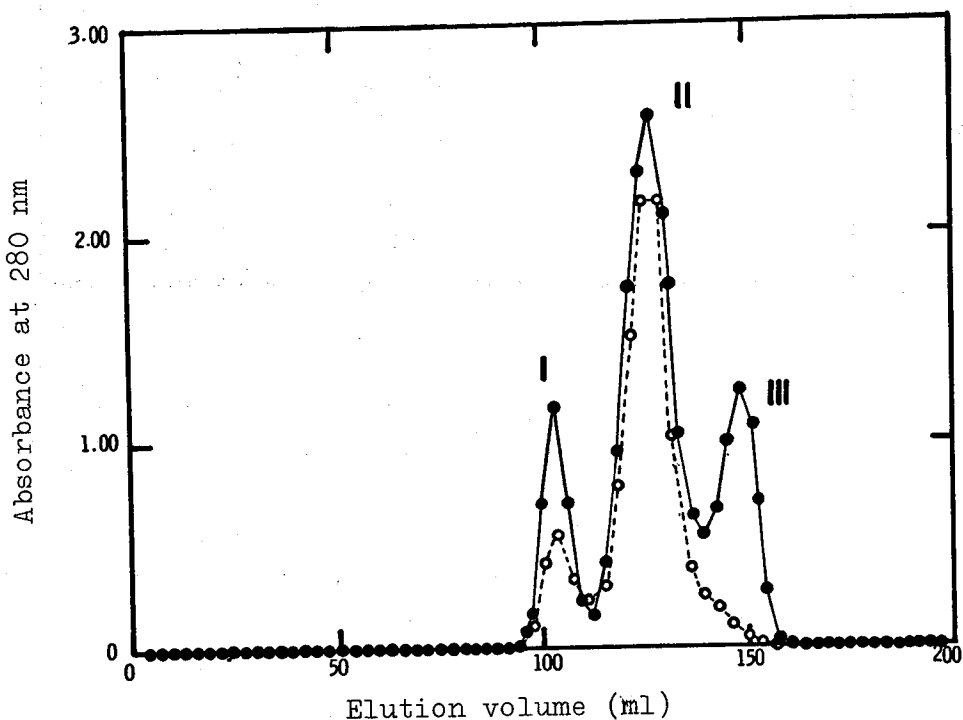


Fig. 5. Gel-filtration of native HEL treated with HF at 0°C for 90 min (—●—) or 270 min (-○-) on Sephadex G-50 (2.2 x 95 cm) using buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

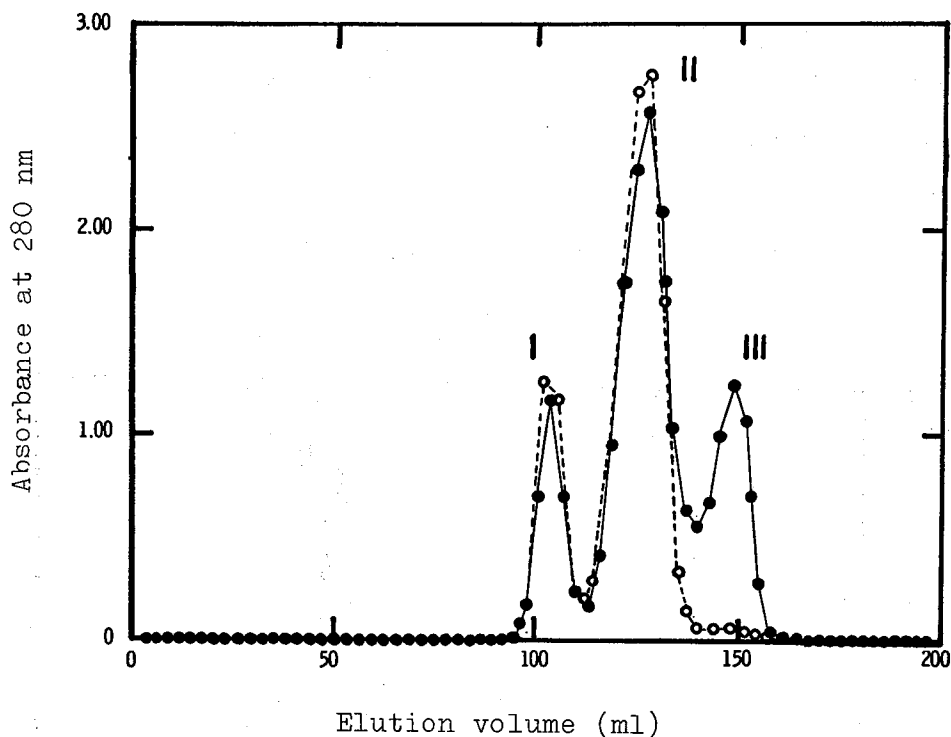


Fig. 6. Gel-filtration of native HEL treated with HF for 90 min at 0°C (—●—●) or 25°C (-○-○-) on Sephadex G-50 (2.2 x 95 cm) using buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

Effects of Various Buffers on the Recovery of Fraction III.

The freeze-dried native HEL was treated with HF at 0°C for 90 min and then kept in various buffers at room temperature for 20 hr. Then the solution was desalted and lyophilized and the residues were subjected to gel-filtration on Sephadex G-50. The recovery of fraction III corresponding to native

HEL was calculated from its absorbance, as shown in Table 1.

Table 1. Effects of various buffers on the recovery of fraction III from HF-treated native HEL

Sodium phosphate buffer		Average recovery of fraction III (%)
mol concn (M)	pH	
0.067	3.5	46
0.067	6.2	47
0.067	8.0	54
0.1	6.2	49
0.1	8.0	55
0.2	6.2	40

Ion-Exchange Chromatography. The freeze-dried native HEL was treated with HF at 0°C for 90 min and then chromatographed on Sephadex G-50. The fraction III obtained was subjected to chromatography on Bio-rex 70 using 0.2M sodium phosphate buffer solution at pH 7.15. This procedure is generally used for purification of crude HEL isolated from natural sources. It was found that fraction III was eluted in the same position as native HEL, as shown in Fig. 7.

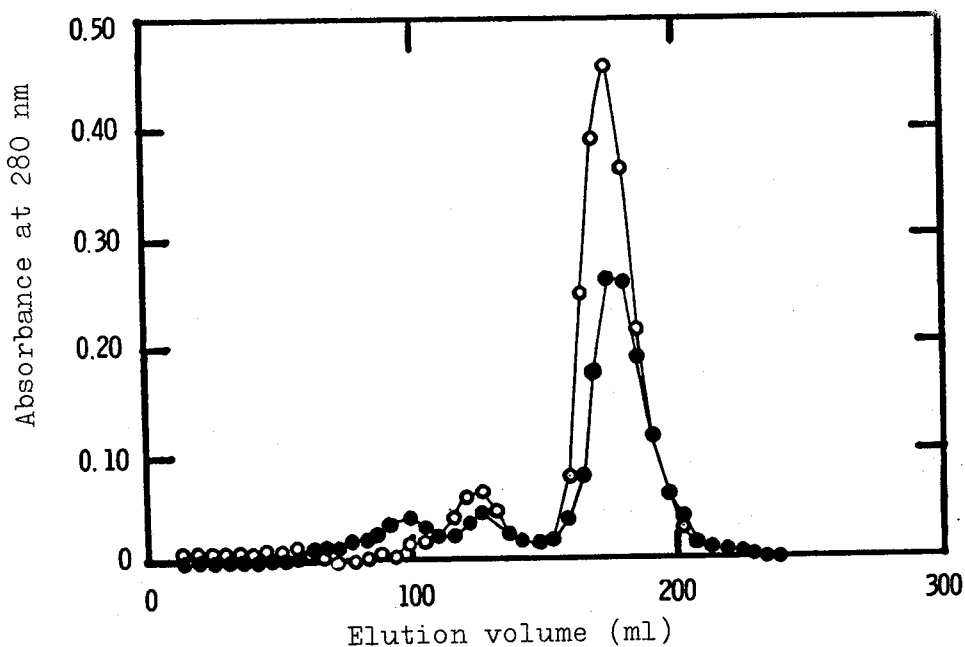


Fig. 7. Ion-exchange chromatogram of HEL on Bio-rex 70 (1.9 x 54 cm) using 0.2M sodium phosphate buffer solution (pH 7.15). The flow rate was 16 ml per hr.

—●— : fraction III of Fig. 2
 —○— : native HEL

Enzymatic Activity of the Fractions of HF-Treated Freeze-dried Native HEL.

The lytic activities of various fractions of HF-treated freeze-dried native HEL, separated by gel- and ionexchange-chromatographies, were determined relative to that of standard HEL on Micrococcus lysodeikticus cell walls. Table 2 shows the specific activities of fractions I, II and III, separated by gel-filtration on Sephadex G-50.

Table 2. Relative activities of fractions I, II and III

HF-treatment	Fraction I	Fraction II	Fraction III
0°C, 90 min	0%	45-66%	97-100%
25°C, 90 min	0	10-15	-

Fraction III consistently showed full specific activity after treatment with HF at 0°C for 90, 120, 150 or 270 min. The main fraction separated by chromatography of fraction III on Bio-rex 70 (Fig.7) was also fully active. However, the fraction II's, separated after treatment of the freeze dried native HEL with HF under different conditions, showed different activities.

Absorption Spectra. Figures 8 and 9 show the ultra-violet absorption spectra of fractions II and III in Fig. 2, respectively. The spectrum of fraction II was slightly different from that of the native HEL, while that of fraction III was the same as that of native HEL. In Fig. 10 the circular dichroism of fraction III of Fig. 2 is compared with that of native HEL. The two spectra are identical in the range of wavelengths measured.

Crystallization of Fraction III. The material, recovered from fraction III in Fig. 2, was kept under conditions inducing

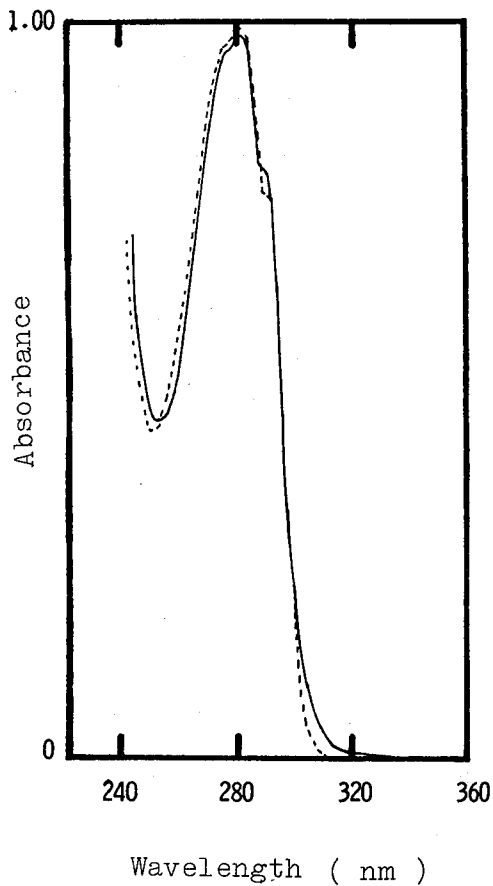


Fig. 8. Ultra-violet absorption spectra of fraction II of Fig. 2 (—) and native HEL (---). Solvent : Buffer containing 1M acetic acid and 5M urea.

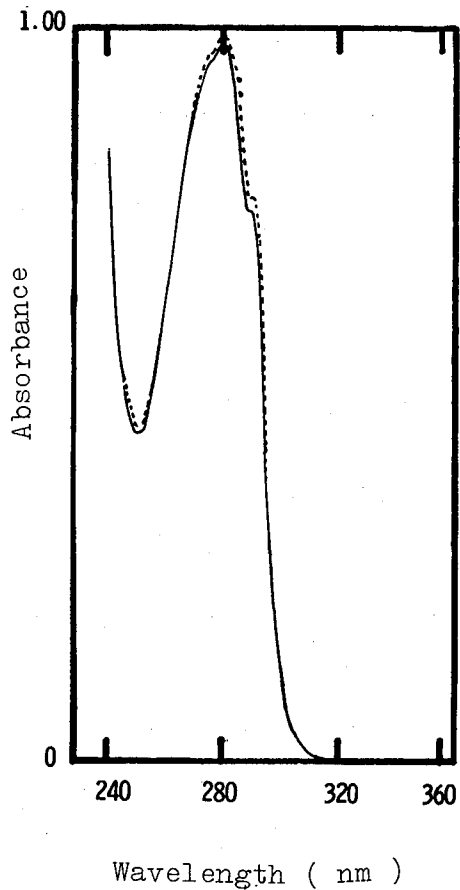


Fig. 9. Ultra-violet absorption spectra of fraction III of Fig. 2 (—) and native HEL (---). Solvent : Buffer containing 1M acetic acid and 5M urea.

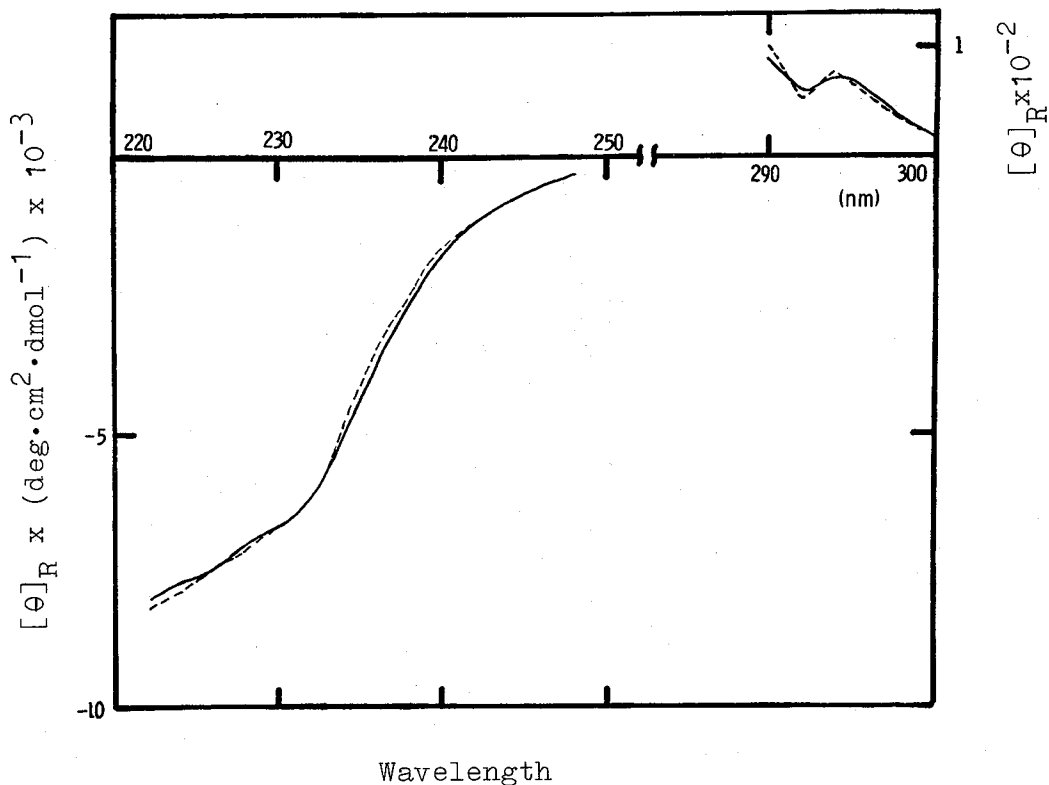


Fig. 10. Circular dichroism spectra of fraction III of Fig. 2 (—) and native HEL (---). Solvent : 0.1M sodium chloride buffer solution adjusted to pH 3.0 with hydrochloric acid.

crystallizations. The crystals obtained were like those of native HEL, as shown in Fig. 11.

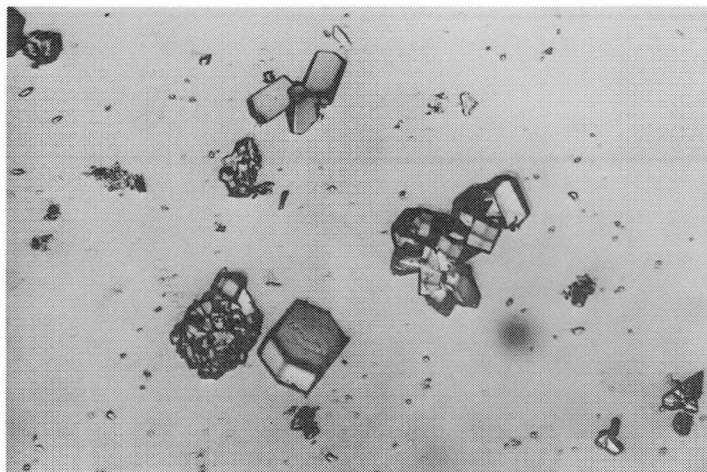


Fig. 11. Microphotograph of crystals of the material from fraction III obtained by the method of Berthou and Jolles.³⁾

Discussion

The enzymatic activities of native HEL decreased during treatment with HF. This suggests that HF may affect structures which are essential for enzymatic activity. After treatment of native HEL with HF three fractions could be separated by gel-filtration in 1M acetic acid and 5M urea buffer. The first fraction eluted, fraction I, was inactive, the second, fraction II, was not fully active and the last, fraction III, was fully active. This suggests that molecules of native HEL were not affected uniformly by treatment with HF, some being only slightly affected and others greatly affected.

This is supported by the finding that on prolongation of the period of treatment of native HEL with HF, the amount of fraction II increased while that of fraction III decreased.

The properties of fractions I, II and III were compared with those of native HEL. Fraction I seemed to contain polymeric materials formed during treatment of native HEL with HF, because it was eluted in the void volume on gel-filtration, and because it was poorly soluble in a buffer and was enzymatically inactive. Fraction II was eluted before native HEL on gel-filtration. The fraction II, separated after treatment of native HEL with HF at 0°C for 90 min, had 45-66% of the activity of native HEL. However, preparations obtained after HF-treatments under different conditions had different activities. Fraction II could be further separated into two fractions, IIa and IIb by gel-filtration in 5% NaCl and 5M urea buffer solution. After treatment of native HEL with HF at 0°C for 90 min, fraction II had 45-66% of the activity of native HEL, while fraction IIa had 2.5% and fraction IIb had about 85% of the activity of native HEL. Chromatograms of fractions IIa and IIb on Bio-rex 70 showed different patterns from that of native HEL. Furthermore, when the material in fraction II was reduced and refolded under the method of Saxena and Wetlaufer,⁴⁾ the resultant material did not move in the position of fraction III on gel-filtration in 1M acetic acid and 5M urea buffer. Thus fraction II was a mixture of at least two components formed by modifications of the structure

of HEL during treatment with HF.

On gel-filtration fraction III was eluted in the same position as native HEL, and it also gave the same pattern on Bio-rex 70 as native HEL. These results suggest that fraction III has the similar properties to native HEL. In addition, the yield of fraction III was about 50% of the initial amount of native HEL. Accordingly, the properties of fraction III were further investigated. The ultra-violet and circular dichroism absorption spectra of fraction III were the same as those of native HEL over the range of wavelengths examined. Moreover, the crystals obtained from fraction III under the same condition used to crystallize native HEL could not be distinguished from the latter.

The results described above indicate clearly that material which was indistinguishable in various properties from native HEL could be recovered in a crystalline state after treatment of native HEL with HF under the usual conditions of peptide synthesis. Investigations on the influence of HF on reduced HEL are described in the next part.

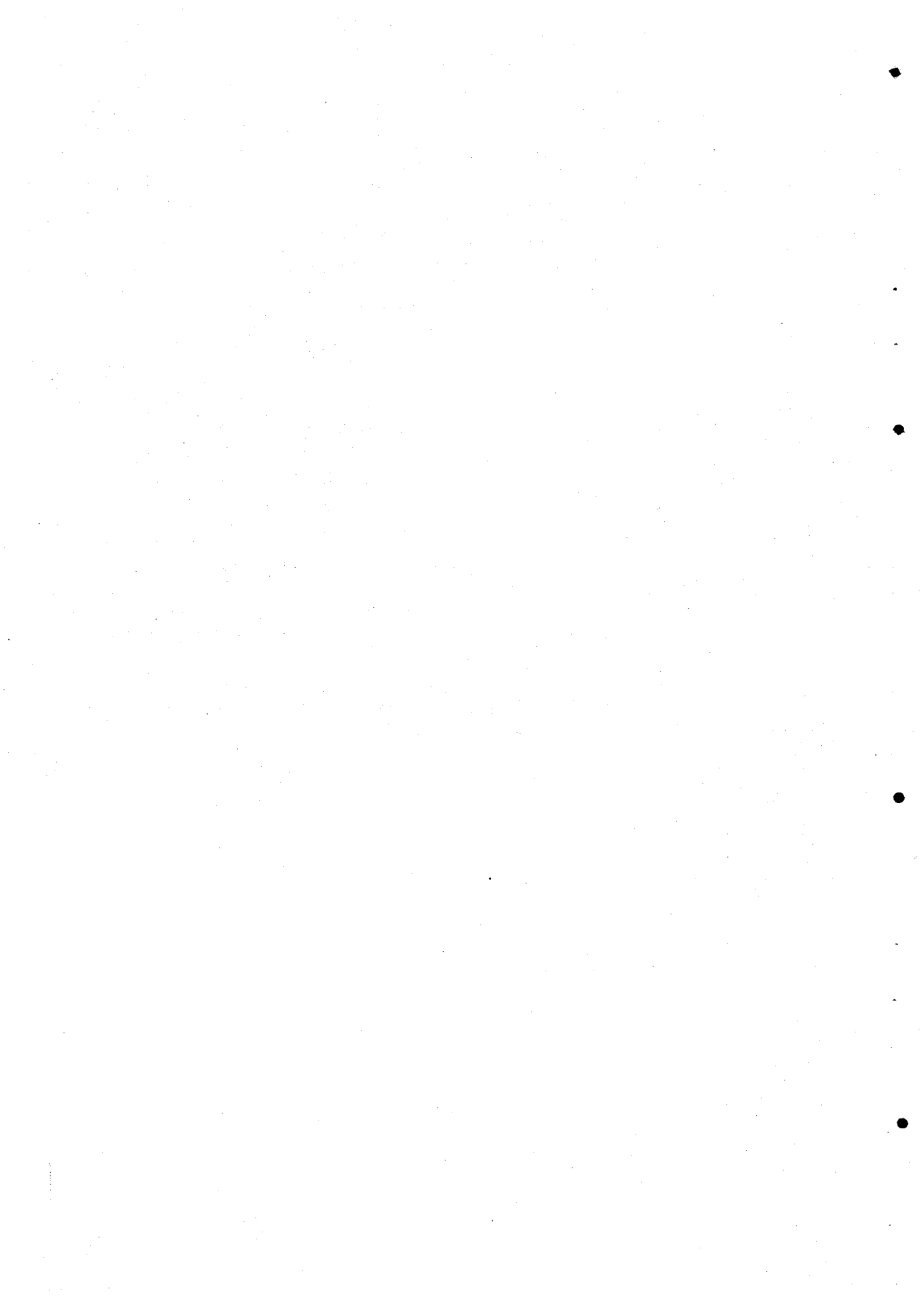
References

- 1) S.Sakakibara, Y.Shimonishi, Y.Kishida, M.Okada, and H. Sugihara, Bull.Chem.Soc.Jpn., 40, 2164 (1967).
- 2) P.Jolles, "Methods in Enzymology", ed. by S.P.Colowick

and N.O.Kaplan, Vol. 5, Academic Press, New York (1962),
p. 137.

3) J.Berthou and P.Jolles, FEBS Lett., 31, 189 (1973).

4) V.P.Saxena and D.B.Wetlaufer, Biochemistry, 9, 5015 (1970).



Part II

Influence of Anhydrous Hydrogen Fluoride on Reduced Hen Egg-White Lysozyme

In part I, the author described the effect of anhydrous hydrogen fluoride (HF) on the native form of hen egg-white lysozyme (HEL) under the usual conditions of peptide synthesis and a method for recovering active HEL from an HF-solution of native HEL. In part II, studies on the influence of HF on the reduced form of HEL are described. First, reduced HEL was treated with HF under usual conditions for peptide synthesis, in comparison with native HEL. Second, the effect of thiol compounds during treatment of reduced HEL with HF was examined, because thiol compounds prevent modifications, such as oxidation of proteins. Finally, studies were made on the treatment of reduced HEL with HF in the presence of amino acid derivatives, which liberate cations on treatment with HF. In the studies, the effect of a scavenger was also investigated.

Materials and Methods

Materials Hen egg-white lysozyme (6 x crystallized Lot. No. 7103) and spray-dried cells of Micrococcus lysodeikticus (Lot. No. 21-88-778) were purchased from Seikagaku Fine Biochemicals (Tokyo). Sephadex was purchased from Pharmacia Co. (Uppsala). Hydrogen fluoride was a product of Daikin

Ind. Co. (Osaka). Urea of reagent grade was recrystallized from ethanol before use. All other chemicals used were of analytical grade.

Treatment of Reduced HEL with HF Reduced HEL was prepared

from native HEL by the method of Saxena and Wetlaufer,¹⁾ and after desalting, it was frozen and lyophilized. The freeze-dried powder of reduced HEL (ca. 15 mg) was treated with HF (9 ml) under similar conditions to those used with native HEL in part I. The residue of the HF-treated reduced HEL was dissolved in 0.2M sodium phosphate buffer solution (15 ml) at pH 8.5 containing 1M mercaptoethanol, 8M urea and 0.2mM EDTA·2Na and the solution was stirred overnight at room temperature. Then pH of the solution was adjusted to below 4 by addition of glacial acetic acid and the solution was passed through a column of Sephadex G-10 (3.2 x 60 cm) in 1M acetic acid. The fractions containing protein were collected and treated under the conditions described below for refolding reduced HEL. Then, the pH of the solution containing regenerated HEL was adjusted to 4.5-5 with glacial acetic acid. The regenerated HEL was adsorbed on a CM-cellulose column (carboxylic acid cycle), and eluted with 0.3M sodium phosphate buffer solution at pH 8.0.

Refolding of Reduced HEL to Native HEL Refolding of re-

duced HEL (ca. 10^{-6} M) to form native HEL was achieved in 0.08M tris buffer solution at pH 8.0 containing 5.4×10^{-3} M L-cysteine and 4.8×10^{-4} M L-cystine at 37°C following the procedure of

Saxcena and Wetlaufer.¹⁾

Gel-filtration and Ion-exchange Chromatography of HF-treated Reduced HEL

HF-treated HEL refolded as described above was chromatographed on Sephadex G-50 (2 x 95 cm) using buffer containing 1M acetic acid and 5M urea, using the method described in part I. The filtrations separated by gel-filtration on Sephadex G-50 were chromatographed on Bio-rex 70 (1 x 78 cm) using 0.2M sodium phosphate buffer solution at pH 7.18.

Enzyme Assays The specific activity of HF-treated HEL was determined by measuring the initial rate of lysis of Micrococcus lysodeikticus cells spectrophotometrically at 540 nm following the method described in part I, with native HEL, purified on Sephadex G-50, as a standard.

Ultra-violet Spectra UV-absorption spectra were measured using a Hitachi spectrophotometer, type-124, equipped with a recording attachment. Spectra were measured at 25°C.

Crystallization of HF-treated HEL Crystallization was carried out by the method of Berthou and Jolles²⁾ with the modifications described in part I.

Results and Discussion

Treatment of Reduced HEL with HF Reduced HEL was first treated with HF at 0°C for 90 min, since it has been found that fully active HEL is recovered in good yield (about 50%),

when native HEL is treated with HF under these conditions. The HF was completely evaporated off from the HF-solution of reduced HEL in vacuo, and the residue was mixed with 0.2M sodium phosphate buffer solution at pH 8.5 containing 1M mercaptoethanol, 8M urea and 0.2 mM EDTA·2Na. This buffer solution was used because it was thought that reduced HEL might aggregate by forming random disulfide linkages in HF and that this buffer solution might dissociate the aggregates, because it has been used for reducing native enzyme.¹⁾ In fact the residue did aggregate. However, the aggregates did not easily dissolve in this buffer solution. The supernatant was separated from aggregated material and desalted and then treated under the conditions used for refolding reduced HEL. The refolded protein was desalted, lyophilized and chromatographed on Sephadex G-50 in 1M acetic acid and 5M urea buffer solution, as shown in Fig. 1. The recovery of fraction II, eluted in the same position as native HEL, corresponded to only 5% of the original enzyme used. The recovery of this fraction was low probably because reduced HEL aggregated to sparingly soluble material on treatment with HF.

Treatment of Reduced HEL with HF in the Presence of Thiol Compounds

It was thought that an appropriate thiol compound may prevent the aggregation of reduced HEL on treatment with HF. Therefore, reduced HEL was treated with HF under the same conditions in the presence of the thiol compounds listed in Table 1.

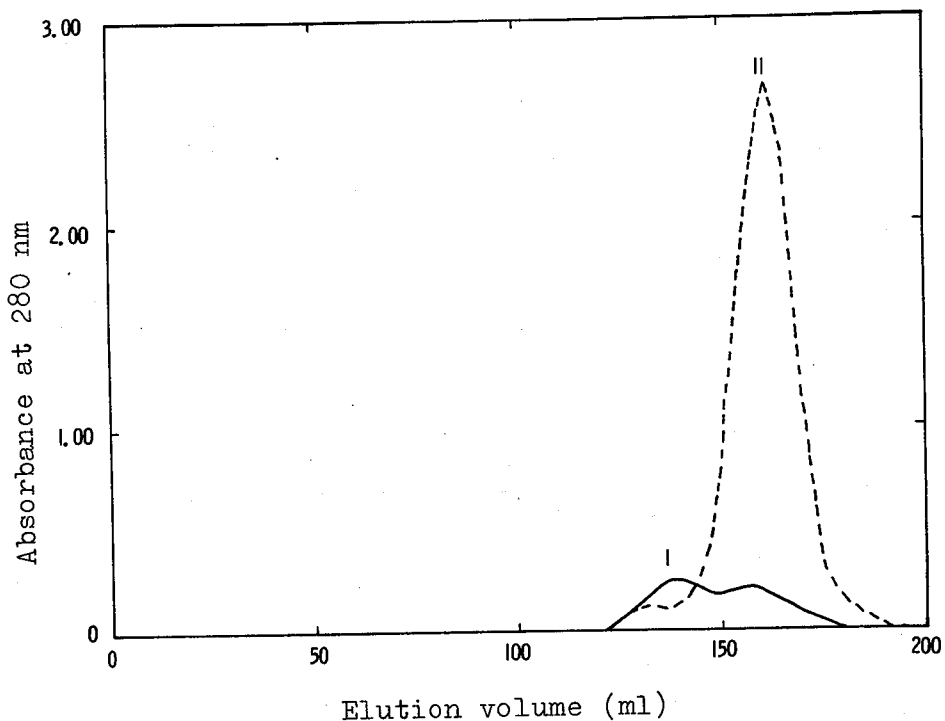


Fig. 1. Sephadex G-50 gel-filtration of reduced HEL after treatment with HF at 0°C for 90 min and refolding at pH 8.0. The column (2.2 x 95 cm) was eluted with buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

— : reduced HEL refolded after HF-treatment
 - - - : reduced HEL refolded without HF-treatment

Table 1. Effects of thiol compounds on the recovery of fraction II from HF-treated reduced HEL

Exp. No.	Thiol Compound	HF-treatment	Recovery of fraction II (%)	Specific activity ^{a)} of fraction II (%)
1	—	— ^{b)}	65	98
2	—	0°C, 90min	5	95
3	HSCH ₂ CH ₂ OH ^{c)}	0°C, 90min	0-1	43
4	L-Cysteine·HCl·H ₂ O ^{c)}	0°C, 90min	24	97
5	L-Cysteine ^{c)}	0°C, 90min	29	98
6	1,4-Butane-dithiol ^{d)}	0°C, 90min	33	99
7	Dithiothreitol ^{d)}	0°C, 90min	19	100

a) 6 x crystallized HEL purified on Sephadex G-50 was used as a standard; b) reduced HEL was refolded without HF-treatment; c) 0.8 mmol of thiol compound was added per umol of protein; d) 0.4 mmol of thiol compound was added per umol of protein.

In this case, the residue of HF-treated reduced HEL dissolved easily in 0.2M sodium phosphate buffer solution containing 1M mercaptoethanol, 8M urea and 0.2mM EDTA·2Na and the aggregation did not appeared. This protein fraction was isolated by gel-filtration on Sephadex G-10, treated under the conditions leading to refolding of reduced HEL and then chromatographed on Sephadex G-50. It was found that the recovery

of fully active HEL, eluted in the same position as native HEL, was improved considerably using L-cysteine or 1,4-butanedithiol as the thiol compound and was improved slightly using dithiothreitol. However, scarcely any fraction II was obtained using mercaptoethanol. The chromatogram of regenerated protein on Sephadex G-50 obtained using 1,4-butanedithiol as a thiol compound is shown in Fig. 2.

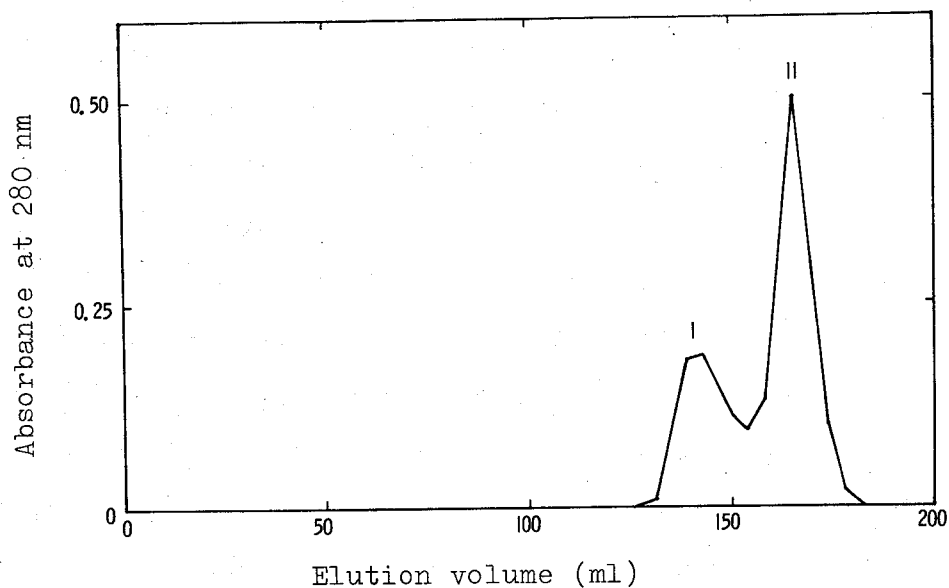


Fig. 2. Sephadex G-50 gel-filtration of reduced HEL after treatment with HEL at 0°C for 90 min in the presence of 1,4-butanedithiol and refolding at pH 8.0. The column (2.0 x 95 cm) was eluted with buffer containing 1M acetic acid and 5M urea. The flow rate was 10 ml per hr.

The amount of fraction II in Fig. 2 corresponds to the recovery shown in Table 1.

Treatment of Native HEL with HF in the Presence of Thiol

Compounds It was found that the aggregation of reduced HEL was extremely prevented on treatment with HF in the presence of thiol compounds. Since native HEL aggregates slightly in HF as described in part I, the effect of the thiol compounds was examined on the treatment of native HEL with HF. Native HEL was treated with HF under the same conditions as reduced HEL in the presence of various thiol compounds. After treatments similar to those used with reduced HEL the regenerated protein was subjected to chromatography on Sephadex G-50 using buffer solution containing 1M acetic acid and 5M urea. The yields of fraction II, eluted at the same position as native HEL, are summarized in Table 2. The recoveries of fully active enzyme from native HEL treated with HF in the presence of 1,4-butanedithiol and L-cysteine were 32 and 36%, respectively, while the recovery of fully active enzyme from native HEL treated with HF in the absence of thiol compounds was 25%. Thus thiol compounds also improved the yield on treatment of Native HEL with HF. Since the recoveries of fully active HEL from reduced and native HELs treated with HF in the presence of the thiol compounds were much higher than those in the absence of the thiol compounds, respectively, the thiol compounds seemed to prevent both these aggregations and also structural modifications of HEL in HF. It was also

found that the presence of L-tryptophan during treatments of native HEL with HF greatly decreased the recovery of fully active HEL under the present conditions.

Table 2. Effects of various compounds on the recovery of fraction II from HF-treated native HEL

Compound	Average recovery of fraction II (%)	Ratio
1) - ^{a)}	65	100
2) None	25	38
3) 1,4-Butanedithiol ^{b)}	36	55
4) L-Cysteine ^{b)}	32	49
5) L-Cysteine·HCl·H ₂ O ^{b)}	32	49
6) HSCH ₂ CH ₂ OH ^{b)}	0	0
7) L-Methionine ^{b)}	34	52
8) L-Alanine ^{c)}	26	40
9) L-Tryptophan ^{d)}	18	28
10) L-Tryptophan ^{e)}	3	5
11) L-Tryptophan ^{f)}	0	0

a) Native HEL was reduced and refolded without HF-treatment. The amounts of compounds added per μ mole of native HEL were; b) 2.5 mmol; c) 4.0 mmol; d) 0.5 mmol; e) 1.8mmol and f) 2.4 mmol.

Treatment of Reduced HEL with HF in the Presence of Amino

Acid Derivatives

Next, the author made preliminary experiments on the removal of protecting groups from the protected derivatives of HEL, the synthesis of which are described in the next part. Reduced HEL was first treated with HF in the presence of anisole, because anisole has generally been used as a scavenger of cations liberated from protected amino acids and peptides on treatment with HF.³⁾ 1,4-Butanedithiol was also added to the reaction mixture, because among the thiol compounds tested it caused the greatest improvement in recovery of fully active enzyme, as shown in Table 1. When reduced HEL was tested with HF at 0°C for 90 min with 1,4-butanedithiol, the recovery of fully active enzyme was 14% in the presence of anisole and 33% in its absence. The reason for this lower recovery in the presence of anisole seemed to be that the excess anisole could not easily be removed from the HF-reaction mixture, so that the HF could not be removed rapidly. On increase in the time of reaction (150-270 min) of reduced HEL with HF at 0°C, the amount of fraction II decreased. Furthermore, the yield of fraction II was very low when reduced HEL was treated with HF at a higher temperature (25°C), even after reaction for only 60 min. These results are summarized in Table 3.

Table 3. Recoveries (%) of fraction II, from reduced HEL (15 mg) treated with HF (9 ml) under various conditions in the presence of 1,4-butanedithiol (0.3 ml) and anisole (1 ml), refolded at pH 8.0 and chromatographed on Sephadex G-50

	60 min	90 min	150 min	270 min
0°C	17	14	4.1	2.4
25°C	1.1	0.5	0.6	0.3

Second, reduced HEL was treated with HF in the presence of anisole, 1,4-butanedithiol and amino acid derivatives, which bind protecting groups and liberate them as cations on treatment with HF. The amino acid derivatives used were N^E-benzyloxycarbonyl-L-lysine, N^α-benzyloxycarbonyl-S-benzyl-L-cysteine and S-p-methoxybenzyl-L-cysteine which liberate the benzyl group on treatment with HF, N^α-benzyloxycarbonyl-N^ω-tosyl-L-arginine which liberates a tosyl group, t-butoxycarbonylglycine which liberates t-butyl group and N^ω-nitro-L-arginine which liberates a nitro group. The results in Table 4 show that most of these amino acid derivatives did not affect the yield of fraction II but N^ω-nitro-L-arginine decreased the yield greatly. The reason for this is unknown.

Table 4. Effects of various amino acid derivatives on the recovery of fraction II from HF-treated reduced HEL

Exp. No.	Amino acid derivative	(mmol) ^{a)}	Recovery of fraction II (%)
1	H-Lys(Z)-OH	1.0	13
2	Z-Cys(Bzl)-OH	0.5	15
3	H-Cys(Bzl(OMe))-OH	1.0	13
4	Z-Arg(Tos)-OH	1.0	16
5	Boc-Gly-OH	1.0	14
6	H-Arg(NO ₂)-OH	1.0	3

a) Reduced HEL (15 mg), 1,4-butanedithiol (0.3 ml), anisole (1 ml) and the indicated amino acid derivatives (0.5 mmol or 1.0 mmol) were mixed and treated with HF (9 ml) at 0°C for 90 min.

Third, reduced HEL was treated with HF at 0°C for 90 min in the presence of the mixture of amino acid derivatives given in Fig. 3. The regenerated fraction was chromatographed on Sephadex G-50 using buffer solution containing 1M acetic acid and 5M urea, as shown in Fig. 3. The amount of fraction II obtained was 33% of the initial amount of enzyme. This yield was better than those obtained on treatment of reduced HEL with HF in the presence of any of the amino acid deriva-

tives alone.

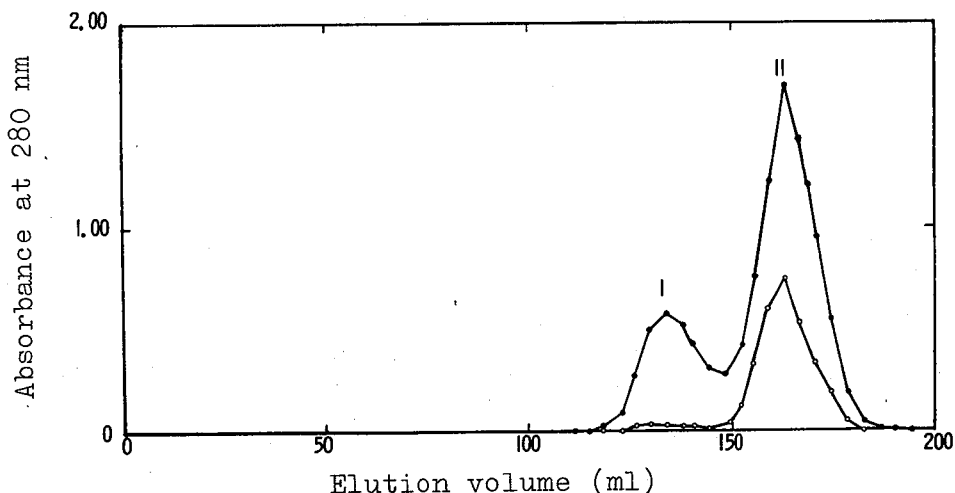


Fig. 3. Sephadex G-50 gel-filtration of reduced HEL treated with HF. The column (2.0 x 95 cm) was eluted with buffer containing 1M acetic acid and 5M urea. Reduced HEL (38 mg) was mixed with anisole (1.5 ml, 14 mmol), 1,4-butanedithiol (1.0 ml, 8.8 mmol), Z-Arg(Tos)-OH (231 mg, 0.5 mmol), H-Cys(Bzl)-OH (106 mg, 0.5 mmol), H-Cys(Bzl(OMe))-OH HCl (130 mg, 0.5 mmol) and Boc-Gly-OH (88 mg, 0.5 mmol), treated with HF (15 ml) and refolded at pH 8.0.

—●—●— : HF-treated reduced HEL

-○-○- : native HEL

When fraction II of Fig. 3 was subjected to chromatography on Bio-rex 70 at pH 7.18, the main fraction eluted in the position of native HEL (Fig. 4) was fully active on Micrococcus lysodeikticus cell walls, and it had the same UV-spectrum as

shown in Fig. 5.

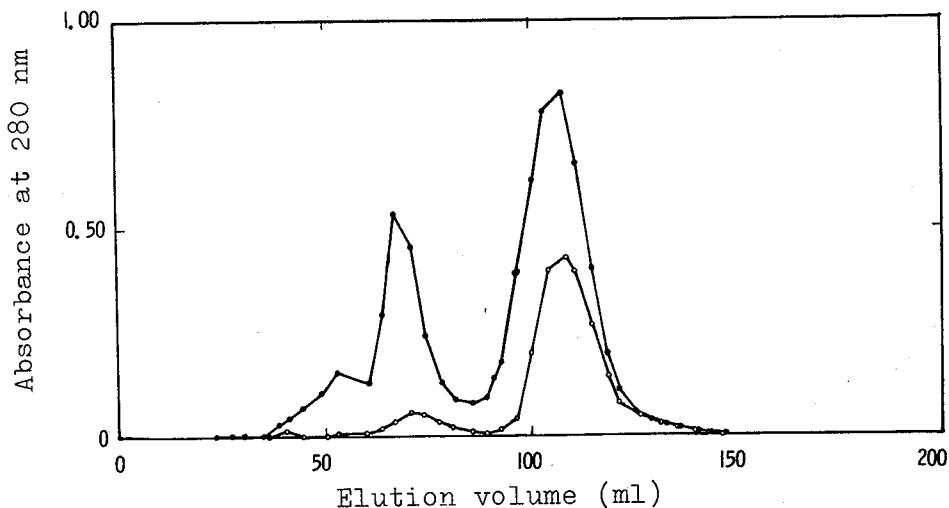


Fig. 4. Ion-exchange chromatogram of HEL on Bio-plex 70. The column (1 x 78 cm) was eluted with 0.2M sodium phosphate buffer solution (pH 7.18). The flow rate was 16 ml per hr.

—●—●— : fraction II of Fig. 3
—○—○— : native HEL

During this chromatography, a fairly large fraction with biological activity was eluted in front of the main fraction, but its properties were not examined. The main fraction in Fig. 4 was desalted on Sephadex G-10 and lyophilized. The lyophilized powder was treated under conditions inducing crystallization. The crystals obtained were like those of native HEL, as shown in Fig. 6.

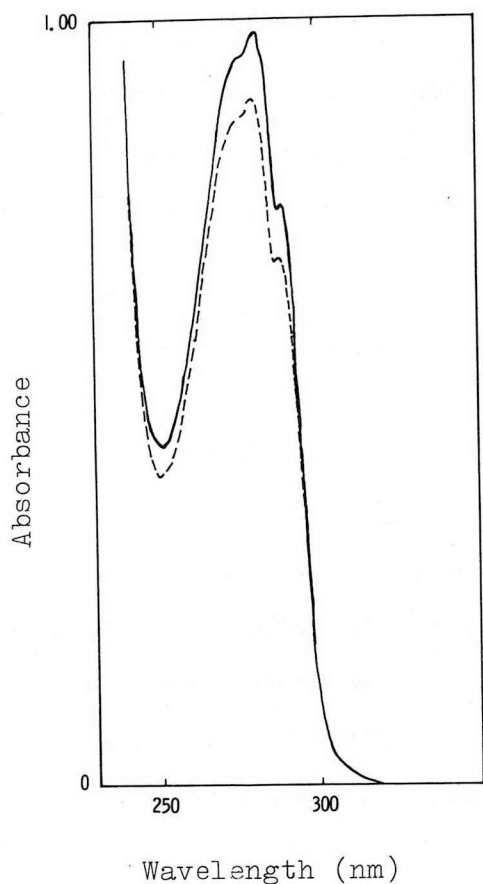


Fig. 5. Ultra-violet absorption spectra of the main fraction of Fig. 4 (—) and native HEL (---). Solvent : 0.2M sodium phosphate buffer solution (pH 7.18).

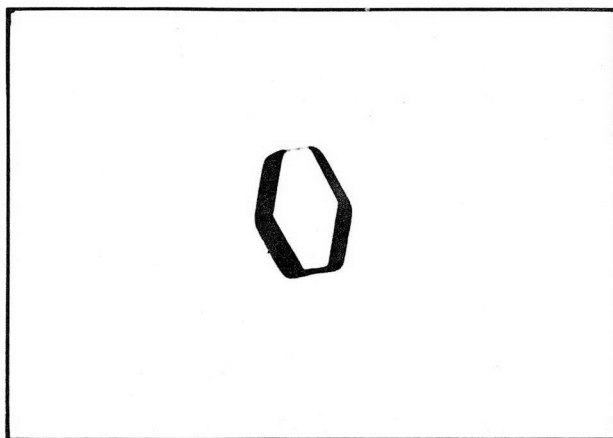


Fig. 6. Microphotograph of crystals of the material from the main fraction of Fig. 4. Crystals were obtained by the method of Berthou and Jolles.²⁾

The present results show that material with similar to original HEL could be recovered in fairly good yield and also in a crystalline state after treatment of reduced HEL with HF

in the presence of a thiol compound such as 1,4-butanedithiol under usual conditions for peptide synthesis. In the next part, attempts are described to recover fully active HEL from the protected derivatives of native and reduced HELs.

References

- 1) V.P.Saxena and D.B.Wetlaufer, *Biochemistry*, 9, 5015 (1970).
- 2) J.Berthou and P.Jolles, *FEBS Lett.*, 31, 189 (1973).
- 3) S.Sakakibara, Y.Shimonishi, M.Okada, Y.Kishida, "Peptides", *proc. 8th European Peptide Symp.*, ed. by H.C.Beyermann, et al., North Hol.and Publishing Co., Amsterdam (1967), p. 44; S.Sakakibara, Y.Shimonishi, Y.Kishida, M.Okada, and H.Sugihara, *Bull.Chem.Soc.Jpn.*, 40, 2164 (1967).

Part III

Regeneration to the Native Form of Hen Egg-White Lysozyme from its Protected Derivatives

Previously the author reported the effect of treatment of hen egg-white lysozyme (HEL) with anhydrous liquid hydrogen fluoride (HF) under the usual conditions for peptide synthesis. The author recovered fully active enzyme in a crystalline state from HF-solutions of the native and reduced forms of HEL. As a further step in our studies on the chemical synthesis of HEL, this part describes the preparation of various protected derivatives of HEL from native HEL and the regeneration of active enzyme from them. The protected derivatives synthesized were similar to protected polypeptides covering the entire sequence of HEL, that must be synthesized to obtain the complete lysozyme molecule. Studies were made on the introduction of protecting groups into HEL using the reagents t-butoxycarbonyl azide (Boc-N₃), benzyl 2,5-dioxopyrrolid-1-yl carbonate (Z-ONSu) and its derivatives with the aromatic ring chlorinated for the amino groups of native HEL, and the reagents 4-methoxybenzyl and 4-methylbenzyl chlorides for the thiol groups of reduced HEL. The later reactions were performed to find suitable protected derivatives of HEL that could be regenerated to the original enzyme by treatment with HF under appropriate conditions for peptide synthesis. The results showed that 2-chlorobenzoyloxycarbonyl

and 4-methylbenzyl substituents are extremely suitable for protecting amino and thiol groups, respectively, and that fully active enzyme can be regenerated from derivatives of HEL that are blocked with these protecting groups.

Materials and Methods

Hen egg-white lysozyme (6 x crystallized lot. E5201) and spray-dried cells of Micrococcus lysodeikticus (Lot. No. 21-88-778) were purchased from Seikagaku Fine Biochemicals (Tokyo). Sephadex was purchased from Pharmacia Co. (Uppsala). Hydrogen fluoride was a product of Daikin Industrial Co. (Osaka). Urea of reagent grade was recrystallized from ethanol before use. Chlorinated benzoic acids of extra pure reagent grade were purchased from Nakarai Chemicals Ltd. (Kyoto). All other chemicals used were of analytical grade. Melting points were measured by the capillary method and are given as uncorrected values. HEL derivatives were hydrolyzed in 6M hydrochloric acid with phenol in sealed tubes for 48 h at 105°C, and amino acids in the hydrolysates were examined in a Hitachi KLA-5 analyzer by the method of Moore et al.¹⁾ The abbreviations used in this text are those recommended by IUPAC-IUB: J. Biol. Chem., 247, 977 (1972). Synthesis of benzyl 2,5-dioxopyrrolid-1-yl carbonate (Z-ONSu). Benzylloxycarbonyl chloride (17.1 g, 100 mmol) and N-hydroxysuccinimide (11.5 g, 100 mmol) were dissolved in dioxane

(200 ml). Pyridine (9.5 g, 120 mmol) was added dropwise to the solution at 8-10°C with stirring, and then the mixture was stirred at room temperature for an hour. The mixture was concentrated in vacuo, and the residue was dissolved in ethyl acetate. The solution was washed with water and dried over anhydrous sodium sulfate. The dried solution was concentrated to a crystalline residue in vacuo, and the material was recrystallized from ethyl acetate and hexane; wt 16.0 g; mp 79,5-81.5°C.

Found: C, 57.74; H, 4.55; N, 5.80%. Calcd for $C_{12}H_{11}O_5N$: C, 57.83; H, 4.45; N, 5.62%.

Synthesis of benzyl 2,5-dioxopyrrolid-1-yl carbonate with the aromatic ring chlorinated. 2-Chlorobenzoic acid was reduced with $LiAlH_4$ in dried ether to give the corresponding alcohol, 2-chlorobenzyl alcohol, in about 90% yield. This 2-chlorobenzyl alcohol (10.0 g, 70 mmol) was added to an ethereal solution (200 ml) of phosgen (25 g). The solution was kept for 20 h at room temperature in a closed vessel. The excess phosgen was removed by passing a stream of dry nitrogen gas through the solution, and the solvent was evaporated off under reduced pressure. The residual oil (2-chlorobenzyl-oxycarbonyl chloride) was dissolved in dioxane (160 ml) with N-hydroxysuccinimide (9.2 g, 80 mmol) and pyridine (7.19 g, 100 mmol) at 8-10°C. The solution was treated in a similar way to that of the benzyl derivative. 2-Chlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate [Z(2-Cl)-ONSu] was obtained in

62% yield; mp 103°C.

Found: C, 50.63; H, 3.51; N, 4.99; Cl, 12.59%. Calcd for $C_{12}H_{10}O_5NCl$: C, 50.81; H, 3.55; N, 4.94; Cl, 12.50%.

The following derivatives were prepared in a similar way.

3-Chlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate

[Z(3-Cl)-ONSu]: mp 129.5-130.5°C. Found: C, 50.74; H, 3.51; N, 5.04; Cl, 12.52%. Calcd for $C_{12}H_{10}O_5NCl$: C, 50.81; H, 3.55; N, 4.94; Cl, 12.50%.

4-Chlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate

[Z(4-Cl)-ONSu]: mp 125°C. Found: C, 50.64; H, 3.52; N, 5.05; Cl, 12.58%. Calcd for $C_{12}H_{10}O_5NCl$: C, 50.81; H, 3.55; N, 4.94; Cl, 12.50%.

2,4-Dichlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate

[Z(2,4-Cl₂)-ONSu]: mp 92-3°C. Found: C, 45.15; H, 2.82; N, 4.52; Cl, 22.30%. Calcd for $C_{12}H_9O_5NCl_2$: C, 45.31; H, 2.85; N, 4.40; Cl, 22.29%.

3,4-Dichlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate

[Z(3,4-Cl₂)-ONSu]: mp 118-9°C. Found: C, 45.08; H, 2.79; N, 4.54; Cl, 22.34%. Calcd for $C_{12}H_9O_5NCl_2$: C, 45.31; H, 2.85; N, 4.40; Cl, 22.29%.

2,6-Dichlorobenzyl 2,5-dioxopyrrolid-1-yl carbonate

[Z(2,6-Cl₂)-ONSu]: mp 127-8°C. Found: C, 45.20; H, 2.84; N, 4.54; Cl, 22.03%. Calcd for $C_{12}H_9O_5NCl_2$: C, 45.31; H, 2.85; N, 4.40; Cl, 22.29%.

Reaction of native HEL with t-butoxycarbonyl azide. A

solution of native HEL (100 mg) in distilled water (1.4 ml)

was mixed with pyridine (0.6 ml) and then t-butoxycarbonyl azide (Boc-N₃) (0.428 g, ca. 3 mmol) in dimethyl sulfoxide (4 ml). The mixture was gently stirred until it became homogeneous, and then kept for 48 h at 38°C without stirring. It was concentrated under reduced pressure and the residue was mixed with a large volume of ethyl acetate. The precipitate formed was collected by centrifugation, dissolved in dimethyl sulfoxide and reprecipitated by adding ethyl acetate. The precipitate was again collected by centrifugation and reprecipitated in the same way several times. Finally, the precipitate was dissolved in dimethyl sulfoxide and dialyzed against dilute aqueous acetic acid and then distilled water. The dialyzed solution was lyophilized and the powder was dried over diphosphorus pentoxide in vacuo. This material is denoted as Boc-HEL.

Reaction of native HEL with benzyl 2,5-dioxopyrrolid-1-yl carbonate and its derivatives. Native HEL was allowed to react with benzyl 2,5-dioxopyrrolid-1-yl carbonate (Z-ONSu) or its derivatives [Z(X)-ONSu] under the same conditions as those used with Boc-N₃. These materials are denoted as Z-HEL and Z(X)-HELs.

Determination of free amino groups in proteins. The free amino groups in proteins were measured by a modification of the method of Moore and Stein²⁾ using ninhydrin solution. The reagent was prepared in the following way: stannous chloride (0.8 g) was dissolved in 0.2M citrate buffer (500 ml) at pH 5.0.

The solution was mixed with a solution of ninhydrin (20 g) in methylcellosolve (500 ml), and the mixture was saturated with nitrogen gas and kept in a closed vessel in a refrigerator. Protein was dissolved in 3% aqueous sodium dodecyl sulfate and the solution was mixed with the above stocked solution (1 ml), heated for 20 min in a vigorously boiling water bath, cooled rapidly to room temperature and mixed with 50% aqueous 2-propanol (5 ml). Then within 15 min, the absorbance of the solution at 570 nm was measured. Protein contents of 3% aqueous sodium dodecyl sulfate solutions were determined by measuring the absorbance at 290 nm. The percentage of free amino groups (F %) in a protein was calculated as a ratio to free amino groups of native HEL using the following equation:

$$F = \frac{\frac{A_{570} \text{ sample}}{A_{290} \text{ sample}}}{\frac{A_{570} \text{ native}}{A_{290} \text{ native}}} \times 100$$

where A_{570} sample and A_{570} native mean the absorbances at 570 nm of the sample protein and native HEL solutions after treatment with ninhydrin solution, and A_{290} sample and A_{290} native mean the absorbances at 290 nm of solutions of sample protein and native HEL in 3% aqueous sodium dodecyl sulfate.

Treatment of Boc-HEL with formic acid. Lyophilized powder (ca. 30 mg) of Boc-HEL was dissolved in 99% formic acid (10 ml). The solution was stood for 24 h at 15°C and then lyophilized. The resulting powder was dissolved in buffer solution (7.9 ml),

consisting of 0.2M tris (3 ml) at pH 8.6, 5% EDTA·2Na (0.3 ml), urea (3.61 g) and β -mercaptoethanol (0.5 ml), under a nitrogen atmosphere, and stirred overnight at room temperature. Then the solution was acidified to below pH 4 with glacial acetic acid and charged on a column of Sephadex G-10 (3 x 60 cm). The eluate containing the protein was added to 0.08M tris buffer solution at pH 8 (1 liter) containing 5.4×10^{-3} M L-cysteine and 4.8×10^{-4} M L-cystine.³⁾ The pH of the solution was adjusted to 8.0 with glacial acetic acid or concentrated ammonia. Then, the solution was kept for over 5 h at 37°C, and the pH of the solution was adjusted to 4.5-5 with glacial acetic acid. The solution was applied to a CM-cellulose column (2 x 30 cm, carboxylic acid cycle). The absorbed protein was eluted with 0.3M sodium phosphate buffer at pH 8.0. The eluate containing the protein was acidified with glacial acetic acid and lyophilized. The lyophilized material was desalted by passage through a column of Sephadex G-10 (3.2 x 60 cm) in 1M acetic acid and fractions containing the protein were collected and lyophilized.

Treatment of native HEL and derivatives of HEL with HF.

Lyophilized powder (ca. 30 mg) of native HEL or its derivatives was weighed with 1,4-butanedithiol (0.5 ml) and anisole (1.0 ml) in the Daiflon cylinder of an HF-reaction apparatus.⁴⁾ The cylinder containing the protein was cooled to -78°C in a dry-ice/methanol bath and HF (10 ml) was distilled into it. The reaction was started by transferring the sample from the

bath at -78°C to a bath at 0°C . The resulting solution was stirred at 0°C for a certain period and then the HF was evaporated off as rapidly as possible from the reaction mixture under reduced pressure. The residue was washed repeatedly with hexane and the amorphous powder obtained was treated in the way used for Boc-HEL.

Reductive alkylations of native HEL with benzyl, 4-methoxybenzyl and 4-methylbenzyl groups in liquid ammonia.

Native HEL was reduced with dithiothreitol in liquid ammonia at its boiling temperature for 3 h by the method of Meienhofer et al.⁵⁾ Then an alkylating reagent was added and the solution was refluxed for 3 h. The ammonia was evaporated off from the reaction mixture through a water aspirator.

When benzyl chloride was used as an alkylating reagent, the residue was kept overnight over concentrated sulfuric acid in vacuo, and then washed successively with ether, ethyl acetate and methanol. The crude material obtained was dissolved in a buffer containing 1M acetic acid and 5M urea, and chromatographed on Sephadex G-75 (3 x 60 cm) in the same buffer. The fractions containing protein were collected, dialyzed against distilled water and lyophilized. The material obtained is denoted as HEL(S-Bzl).

When 4-methoxybenzyl and 4-methylbenzyl chlorides were used as alkylating reagents, the residues were dissolved in dimethyl sulfoxide, and ethyl acetate was added to the solutions. The insoluble material formed was collected by

centrifugation, washed repeatedly with ethyl acetate, and then dissolved in dimethyl sulfoxide. The resulting solutions were dialyzed against aqueous acetic acid and then distilled water. The dialyzed solutions were lyophilized and these materials are denoted as HEL(S-Bzl(OMe)) and HEL(S-Bzl(Me)), respectively.

Reductive alkylations of Z(2-Cl)-HEL with 4-methoxybenzyl and 4-methylbenzyl groups in liquid ammonia. Z(2-Cl)-HEL was reduced with dithiothreitol in liquid ammonia and allowed to react with 4-methoxybenzyl or 4-methylbenzyl chloride under similar conditions to those used with native HEL. The crude products were purified by a similar procedure to that used in the preparations of HEL(S-Bzl(OMe)) and HEL(S-Bzl(Me)). The purified products are denoted as Z(2-Cl)-HEL(S-Bzl(OMe)) and Z(2-Cl)-HEL(S-Bzl(Me)), respectively.

Determination of remaining thiols in proteins. The amount of thiols in HEL(S-Bzl) that remained to be alkylated was calculated from the amount of S-benzylcysteine in the acid hydrolysate of the protein, determined by amino acid analysis. On the other hand, the amounts of thiols that remained to be alkylated in HEL(S-Bzl(OMe)), HEL(S-Bzl(Me)), Z(2-Cl)-HEL(S-Bzl(OMe)) and Z(2-Cl)-HEL(S-Bzl(Me)) were calculated from the amount of S-carboxymethylcysteine measured as follows:

Lyophilized powder (ca. 15 mg) of the proteins was dissolved in buffer solution consisting of 0.2M tris (1.2 ml) at pH 8.6, 5% EDTA·2Na (0.12 ml) and guanidine hydrochloride (2.87 g).

The solution was diluted with 0.2M tris (1.5 ml) at pH 8.6 and 5% EDTA·2Na (0.15 ml), mixed with dithiothreitol (24.6 mg) and stirred overnight at room temperature under a nitrogen atmosphere. Then monoiodoacetic acid (270 mg) in 1M NaOH (1 ml) was added. The mixture was stirred for 15 min in a dark room, acidified with formic acid and dialyzed against distilled water, and the dialyzed solution was lyophilized. The resulting powder was hydrolyzed with 6M hydrochloric acid, and the ratios of the amount of S-carboxymethylcysteine to the average amounts of glycine and alanine in the hydrolysate were determined by amino acid analysis.

Treatments of the reduced and alkylated derivatives of HEL with HF. Reductively alkylated derivatives of HEL were treated with HF as described above.

Gel-filtration and ion-exchange chromatography. Gel-filtration and ion-exchange chromatography were used to isolate fully active HEL from the derivatives of HEL treated with HF, as described in the preceding part. Materials were detected by measuring the absorption of the eluate at 280 nm.

Enzyme assays. The specific activity of HEL was determined by measuring the initial rate of lysis of Micrococcus lysodeikticus cell walls spectrophotometrically at 540 nm by the method described in the preceding part, with native HEL as a standard.

Ultra-violet absorption spectra. UV-absorption spectra were measured using a Hitachi spectrophotometer type-124,

equipped with a recording attachment.

Crystallization of HEL. Crystallization was carried out by the method of Berthou and Jolles⁶⁾ with the modification described in the preceding part.

Results

Reaction of native HEL with Boc-N₃. When native HEL was treated with Boc-N₃ in the presence of pyridine in a mixture of dimethyl sulfoxide and water, only 76% of its amino groups were acylated with Boc groups, as estimated by the ninhydrin reaction. However, when the partially acylated native HEL was treated with Boc-N₃ repeatedly under the same condition, but without water, 98% of its amino groups could be acylated.

Reactions of native HEL with Z-ONSu and Z(X)-ONSu. Native HEL was treated with Z-ONSu in ratios of 1.3 to 65 equivalents of the reagent to one amino group of native HEL, then the product was isolated and its acylated amino groups were estimated by the ninhydrin color reaction. The relation between the ratio of Z-ONSu to amino group of native HEL and the percentage of amino groups acylated with Z groups is summarized in Table 1. Native HEL was also treated with the derivatives of Z-ONSu [Z(X)-ONSu] using a ratio of 6.5 equivalents of the reagents to one amino group of native HEL. Measurements with ninhydrin showed that these derivatives cause almost complete acylation of the amino groups with Z(X) groups, as seen in Table 2.

Table 1. Effect of the ratio of equivalents of Z-ONSu to amino groups of native HEL on the percentage of amino groups acylated

	A	B	C	D	E	F
Z-ONSu (equivalents)	1.3	2.6	6.5	13.0	26.0	65.0
Acylated amino groups (%)	95.5	96.6	98.0	99.0	98.4	98.3

Table 2. Percentages of acylated amino groups in Z(X)-HELs

X in Z(X)-ONSu	Acylated amino groups (%)
2-Cl	98.0
3-Cl	98.0
4-Cl	98.0
2,4-Cl ₂	97.0
3,4-Cl ₂	97.0
2,6-Cl ₂	97.0

Removal of Z groups from Z-HEL by treatment with HF.

Z-HEL was treated with HF at 0°C for periods of 0 to 120 min, and then the reaction was stopped by evaporating the HF off

completely. The residue was dissolved in 3% aqueous sodium dodecyl sulfate. The protein concentration in the solution was determined by measuring the optical density at 290 nm and free amino groups in the protein regenerated by HF-treatment were determined by the ninhydrin method. The recovery of regenerated amino groups was calculated from the equation given in the methods, and the results are shown in Fig. 1.

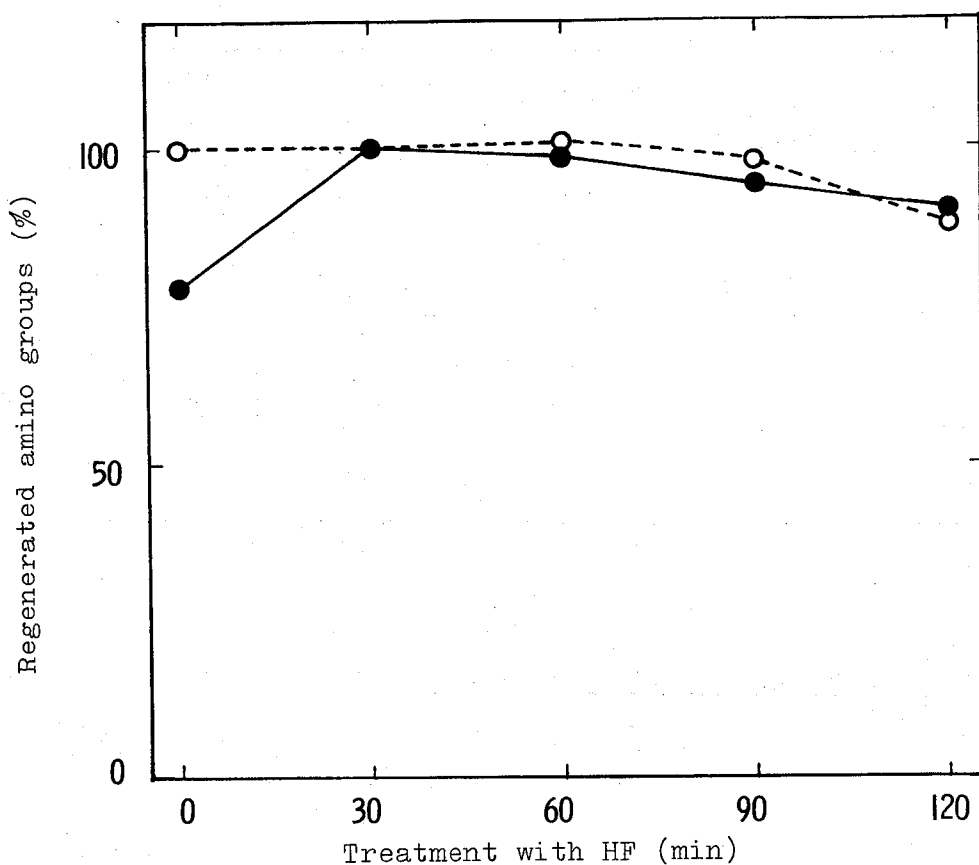


Fig. 1. Removal of Z groups from Z-HEL by HF-treatment.

●—● : Z-HEL, ○—○ : Native HEL

Removal of Z(X) groups from Z(X)-HELs by treatment with HF.

Z(X)-HELs were treated with HF at 0°C for periods of 0 to 120 min, and the free amino groups of the proteins recovered were estimated. The results are shown in Table 3.

Table 3. Percentage of free amino groups recovered from Z(X)-HELs by HF-treatment

Z(X)-HEL	HF-treatment at 0°C (min)				
	0	30	60	90	120
2-Cl	21	96	98	94	83
3-Cl	18	62	97	89	92
4-Cl	74	94	92	90	75
2,4-Cl ₂	18	94	95	83	82
3,4-Cl ₂	13	58	99	92	75
2,6-Cl ₂	18	60	93	90	92

Regeneration to native HEL from Boc-HEL. Treatment of Boc-HEL with formic acid for 24 h at 15°C or with HF for 90 min at 0°C gave fully active HEL in yields of 46 and 25%, respectively. After treatment of native HEL under the same conditions as Boc-HEL, fully active HEL was recovered in yields of 72 and 50%, respectively.

Regeneration to native HEL from Z-HEL. Z-HEL, prepared

under condition C in Table 1, was treated with HF at 0°C for periods of 30 to 90 min. Then the residues of HF-treated Z-HEL were reduced and reoxidized, as described in the methods and the resultant protein fraction was chromatographed on Sephadex G-50 using buffer solution containing 1M acetic acid and 5M urea. The fraction eluted in the same position as native HEL was fully active. After treatment of Z-HEL with HF for 30 min at 0°C the recovery of this fraction was 41%. The recovery of fully active HEL decreased with increase in the period of HF-treatment and after periods of 60 and 90 min the yields of fully active HEL were 28 and 23%, respectively. When the Z-HELs prepared under the various conditions described in Table 1 were treated with HF for 30 min at 0°C, the recoveries of fully active HEL varied depending on the conditions used for preparation of the Z-HELs. The relation between the recoveries of fully active HELs and the conditions used for preparation of Z-HEL are shown in Fig. 2.

Regeneration to native HEL from Z(X)-HELs. Z(X)-HEL was treated with HF for 60 and 90 min at 0°C. Then the HF-treated Z(X)-HEL was reduced, reoxidized and chromatographed on Sephadex G-50, as described in the methods. Two fractions (I and II) were eluted from the column: the first (I) had less enzymatic activity than native HEL, whereas the second (II), eluted in the same position as standard native HEL,

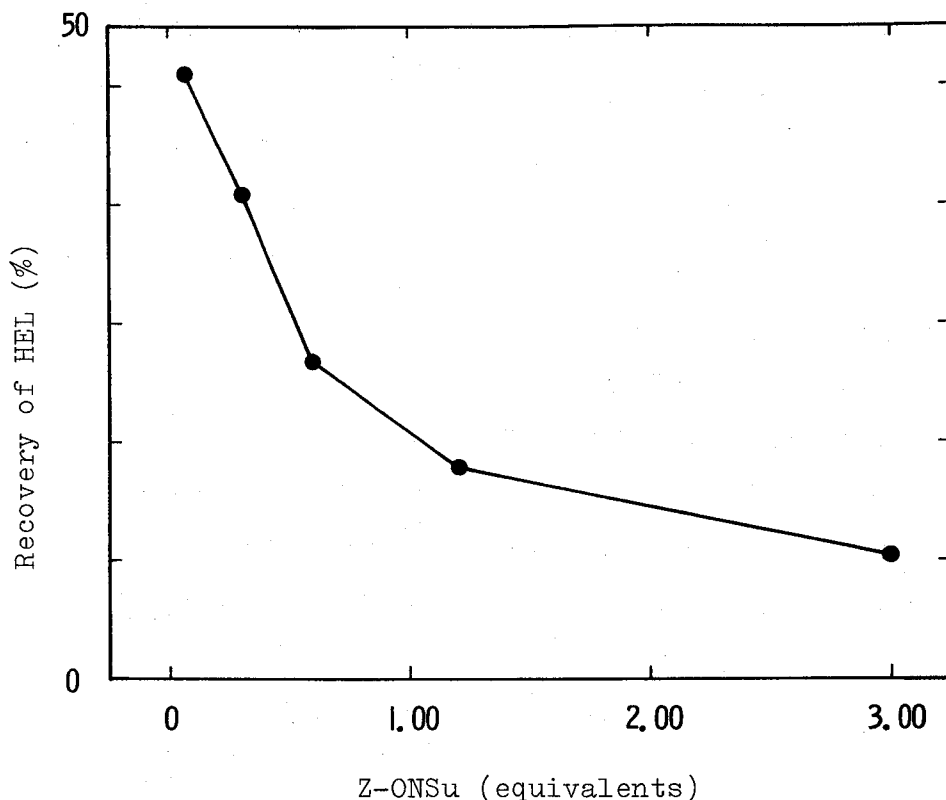


Fig. 2. Effect of the ratio of equivalents of Z-ONSu to amino groups of native HEL used in the synthesis of Z-HEL on the recovery of fully active HEL from Z-HEL by HF-treatment.

had full enzymatic activity. A chromatogram of the protein fraction recovered after HF-treatment of Z(2-Cl)-HEL for 60 min at 0°C is shown in Fig. 3. The recoveries of fraction II, s from Z(X)-HELs are summarized in Table 4.

Purification and crystallization of fully active HEL regenerated from Z(2-Cl)-HEL. Fraction II in Fig. 3 was desalted

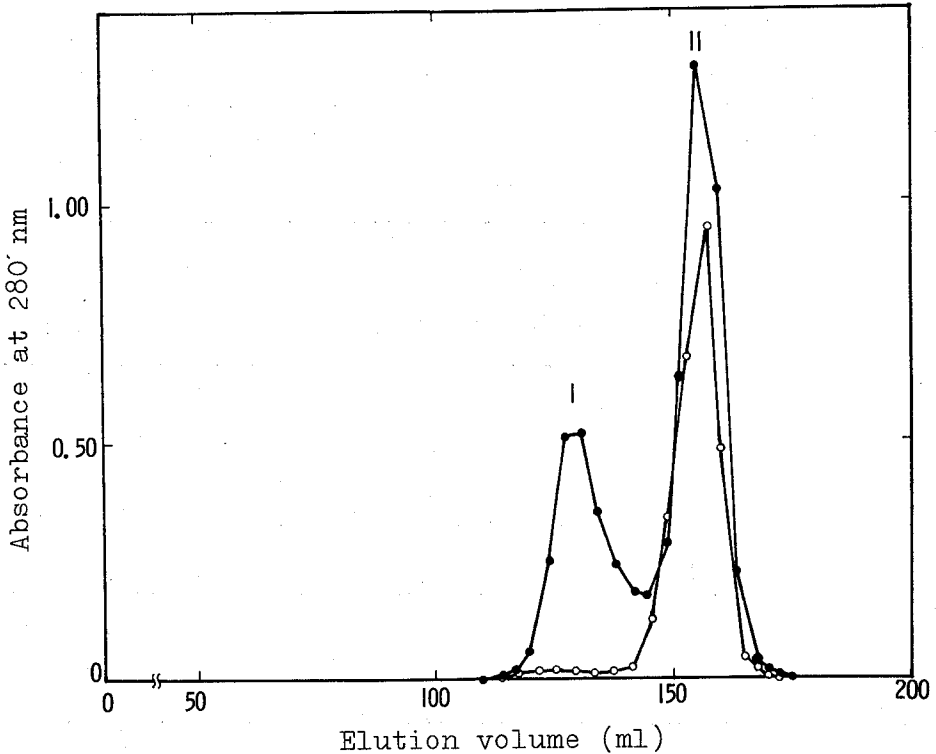


Fig. 3. Gel-filtration of Z(2-Cl)-HEL treated with HF for 60 min at 0°C (—●—●—) and native HEL (—○—○—) on Sephadex G-50 (2 x 95 cm) using buffer containing 1M acetic acid and 5M urea.

by passage through Sephadex G-10, and the eluate containing protein was lyophilized. Then the powder was subjected to chromatography on Bio-rex 70 in 0.2M sodium phosphate buffer solution, pH 7.18, as shown in Fig. 4. In this way four fractions (II-1, II-2, II-3 and II-4) were separated. Fraction II-4 was eluted in the same position as the main fraction of native HEL. The lytic activities of the four fractions,

Table 4. Recoveries of fully active

Conditions for HF-treatment	Native HEL	Z-HEL	2-Cl
0°C, 60 min	59	28	22
0°C, 90 min	50	23	23

Table 5. Reductive alkylations of native HEL

Product	Starting material	(mg)	(μ mol)
HEL(S-Bzl)	native HEL	1000	67
HEL(S-Bzl(OMe))	"	300	20
HEL(S-Bzl(Me))	"	200	13
Z(2-Cl)-HEL(S-Bzl(OMe))	Z(2-Cl)-HEL	120	8
Z(2-Cl)-HEL(S-Bzl(Me))	"	240	16

*) DTT: Dithiothreitol

II-1, II-2, II-3 and II-4, were 65, 96, 101 and 101%, respectively, of that of native HEL in M/15 sodium phosphate buffer solution containing 0.1% sodium chloride, and 18, 29, 48 and 100%, respectively, of that of native HEL in the same buffer

HEL from Z(X)-HELs by HF-treatment

3-Cl	4-Cl	Z(X)-HEL		
		2,4-Cl ₂	3,4-Cl ₂	2,6-Cl ₂
10	20	16	7.3	16
18	26	14	9.5	16

and Z(2-Cl)-HEL

Liq.NH ₃ (liter)	DTT [*] (mmol)	Alkylating reagent	(mmol)	Alkylated SH groups (residue mol)
2.0	5.3	Bzl-Cl	26.4	8.16
2.0	2.0	Bzl(OMe)-Cl	20.0	6.80
1.0	1.1	Bzl(Me)-Cl	11.0	7.12
1.0	1.0	Bzl(OMe)-Cl	10.0	7.36
1.8	2.0	Bzl(Me)-Cl	19.0	7.36

solution containing 1% sodium chloride. The UV absorption spectrum of fraction II-4 is shown in Fig. 5. Fraction II-4 was desalted by passage through Sephadex G-10, and the eluate containing protein was collected and lyophilized. When the

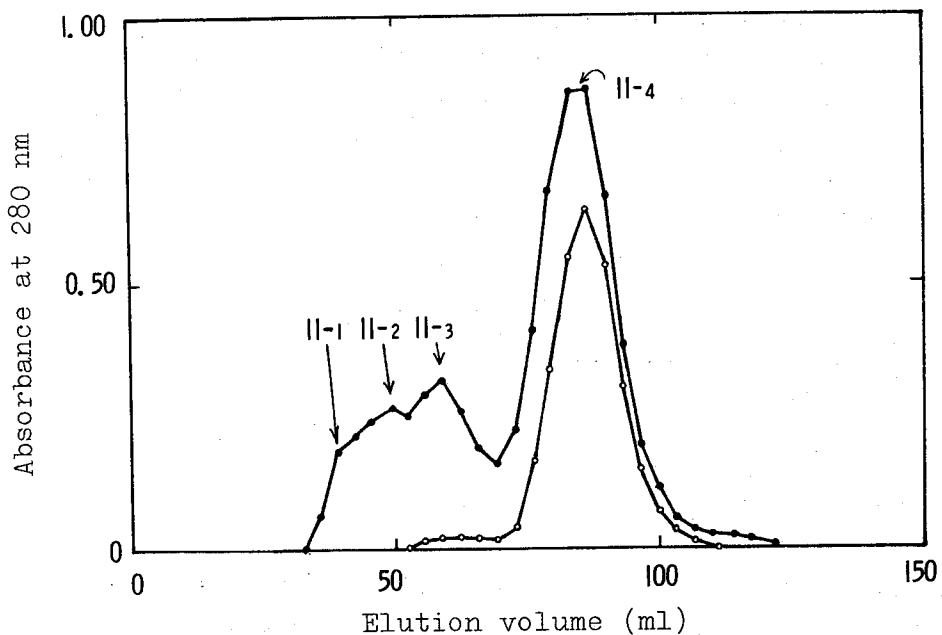


Fig. 4. Ion-exchange chromatogram of fraction II of Fig. 3 (—●—●—) and native HEL (—○—○—) on Bio-rex 70 (1.5 x 33 cm) in 0.2M sodium phosphate buffer solution (pH 7.18)

lyophilized material was stood under conditions inducing crystallization, it formed crystals identical with those of native HEL.

Reductive alkylations of native HEL with benzyl, 4-methoxybenzyl and 4-methylbenzyl groups in liquid ammonia.

Native HEL was reduced and alkylated under the conditions described for Table 5. The degree of alkylation with benzyl groups was estimated from the ratio of the amounts of S-benzylcysteine to the average amounts of glycine and alanine, deter-

mined by amino acid analysis of the acid hydrolysate of the alkylated product, as shown in Table 5. It was difficult

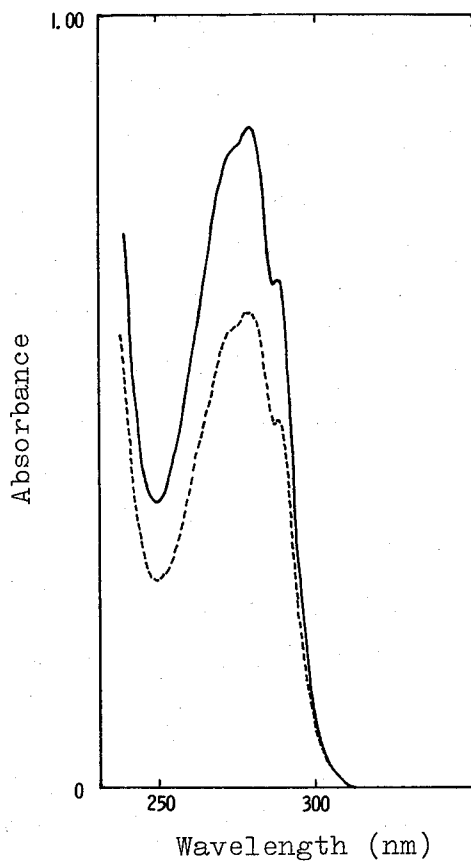


Fig. 5. Ultra-violet absorption spectra of fraction II of Fig. 4 (—) and native HEL (-----). Solvent: 0.2M sodium phosphate buffer (pH 7.18).

to estimate the degree of alkylation with 4-methoxybenzyl and 4-methylbenzyl groups by measuring S-4-methoxybenzylcysteine and S-4-methylbenzylcysteine in acid hydrolysates of the alkylated products by amino acid analysis, because these S-4-methoxybenzyl- and S-4-methylcysteine residues are destroyed during acid hydrolysis. Therefore, they were estimated by analysis of the amount of thiols in the protein that remained unalkylated; namely, the reduced and alkylated derivatives of HEL were carboxymethylated, and the amounts of S-carboxymethylcysteine residues in the carboxymethylated proteins were determined by measuring the amounts of amino acids in their acid hydrolysates with an amino acid analyzer.

The results are summarized in Table 5.

Treatment of HEL(S-Bzl) with HF. HEL(S-Bzl) was treated with HF under various conditions, the resultant material was hydrolyzed with 6M hydrochloric acid, and S-benzylcysteine in the acid hydrolysate was measured by amino acid analysis. Table 6 shows the percentages of S-benzyl groups cleaved by treatment of HEL(S-Bzl) with HF under various conditions. The products of the reactions were treated under similar conditions to those used to isolate enzymatically active HEL from the products of Z-HEL. However, no enzymatically active material was recovered under any of the conditions examined, as shown in Table 7.

Table 6. Percentages of S-benzyl groups cleaved by HF-treatment of HEL(S-Bzl)

	90 min	120 min	150 min
-75°C	10	1	2
-25°C -15°C	4	2	5
0°C	20	32	35

Table 7. Recoveries of fully active HEL from HF-treated HEL(S-Bzl)

	90 min	120 min	150 min
-75°C	0	0	0
-25°C -15°C	0	0	0
0°C	0	0	0

Treatments of S-4-methoxybenzyl and S-4-methylbenzyl derivatives of native HEL and Z(2-Cl)-HEL with HF. The derivatives of HEL were treated with HF at 0°C for 60 and 90 min, and the materials isolated from their HF-solutions were treated

under similar conditions to those described above. The percentages of fully active HEL recovered from them are shown in Table 8.

Table 8. Recoveries of fully active HEL from HEL (S-Bzl(OMe)), HEL(S-Bzl(Me)), Z(2-Cl)-HEL(S-Bzl(OMe)) and Z(2-Cl)-HEL(S-Bzl(Me)) by HF-treatments

Material	HF-treatment (%)	
	0°C, 60 min	0°C, 90 min
HEL(S-Bzl(OMe))	0	1.6
HEL(S-Bzl(Me))	19	12
Z(2-Cl)-HEL(SH)	20	15
Z(2-Cl)-HEL(S-Bzl(OMe))	0	0.5
Z(2-Cl)-HEL(S-Bzl(Me))	8.3	7.4

Purification and crystallization of fully active HEL regenerated from Z(2-Cl)-HEL(S-Bzl(Me)). The material obtained from Z(2-Cl)-HEL(S-Bzl(Me)) by HF-treatment for 60 min at 0°C was reduced, and reoxidized under similar conditions to those used for Z(2-Cl)-HEL. The reoxidized material was chromatographed on Sephadex G-50 using buffer containing 1M acetic acid and 5M urea, as shown in Fig. 6.

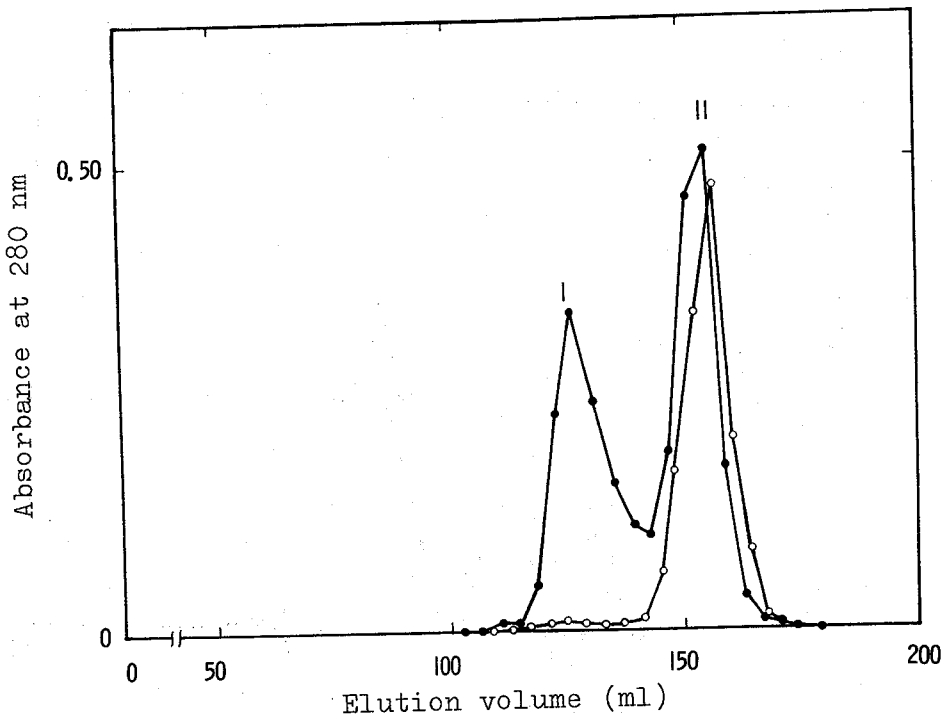


Fig. 6. Gel-filtration of Z(2-Cl)-HEL(S-Bzl(Me)) treated with HF for 60 min at 0°C (—●—●—) and native HEL (—○—○—) on Sephadex G-50 (2 x 95 cm) in buffer containing 1M acetic acid and 5M urea.

Fraction II, eluted in the same position as native HEL, was fully active. The yield of fraction II was 8.3% on the basis of the protein content of the Z(2-Cl)-HEL(S-Bzl(Me)) preparation used, as shown in Table 8. Lyophilized material was obtained from fraction II by desalting and chromatographed on a Bio-rex 70 column using 0.2M sodium phosphate buffer solution at pH 7.18. Fraction II was separated into four

fractions (II-1, II-2, II-3 and II-4), as shown in Fig. 7.

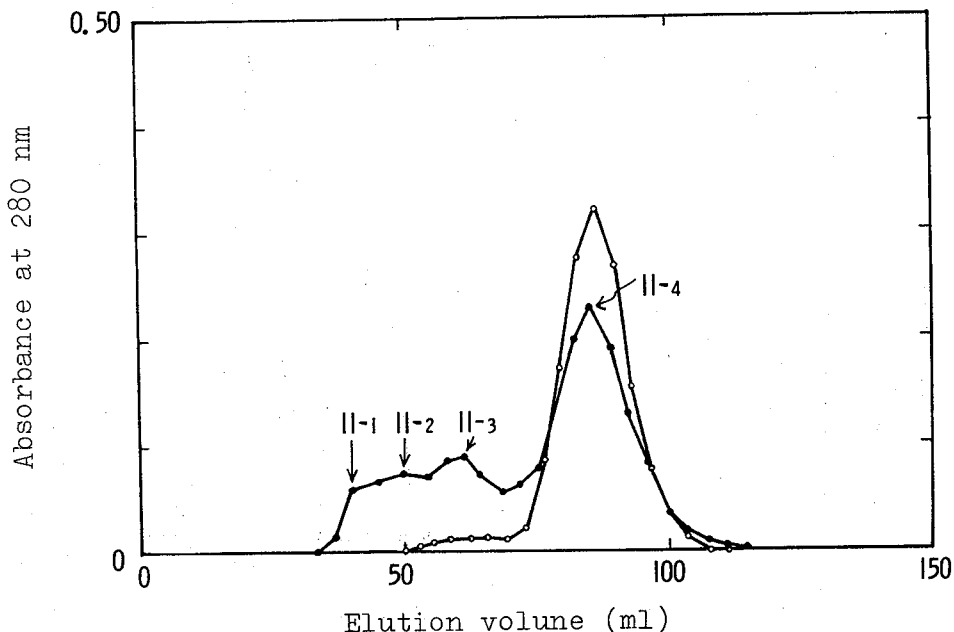


Fig. 7. Ion-exchange chromatogram of fraction II of Fig. 6 (●-●-●) and native HEL (o-o-o) on Bio-rex 70 (1.5 x 33 cm) in 0.2M sodium phosphate buffer solution (pH 7.18).

Fraction II-4 was eluted in the same position as the main fraction of native HEL. These four fractions, II-1, II-2, II-3 and II-4, had 79, 99, 101 and 102%, respectively, of the lytic activity of native HEL in buffer containing 0.1% sodium chloride, and 13, 30, 39 and 102% of the latter in buffer containing 1% sodium chloride. The UV absorption spectrum of fraction II-4 is shown in Fig. 8. When the lyo-

phylized powder obtained from fraction II-4 (Fig. 7) after desalting was stood under conditions inducing crystallization, it yielded similar crystals to those of native HEL, as shown in Fig. 9.

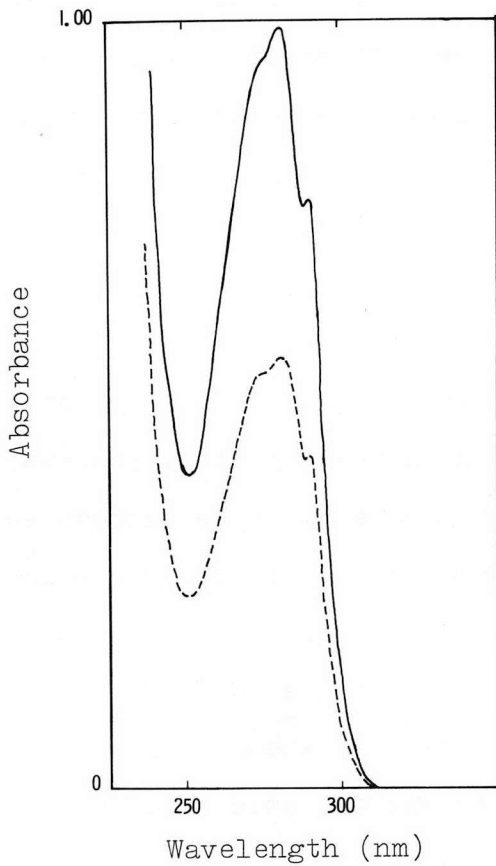


Fig. 8. Ultra-violet absorption spectra of fraction II-4 of Fig. 7 (—) and native HEL (-----). Solvent: 0.2M sodium phosphate buffer (pH 7.18).

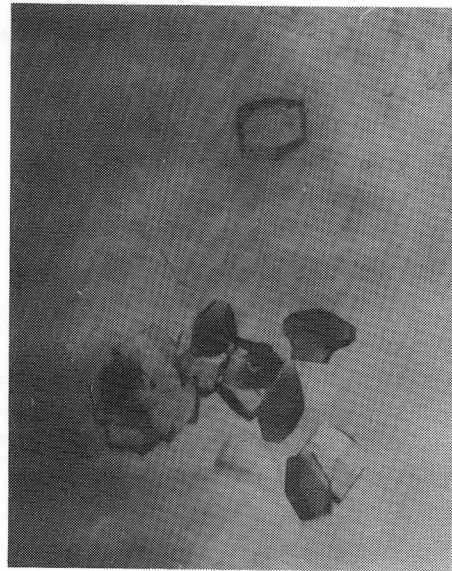


Fig. 9. Microphotograph of crystals of the material from fraction II-4 of Fig. 7.

Discussion

In this work we first prepared derivatives of native HEL in which the amino groups were combined with t-butoxycarbonyl (Boc) or benzyloxycarbonyl (Z) groups or derivatives of the latter, [Z(X)]. These derivatives first were prepared by treating the enzyme with Boc-N₃ or benzyloxycarbonyl azide (Z-N₃). These reagents were not very reactive so that repeated treatments were required to achieve complete acylations of the amino groups of native HEL. Therefore, we next tested Z-ONSu, which can easily be prepared by the reaction of benzyloxycarbonyl chloride (Z-Cl) with N-hydroxysuccinimide (HONSu), instead of Z-N₃ as an acylating reagent and found that with this reagent Z groups could be introduced almost quantitatively onto the amino groups of native HEL in a single reaction. We also found that the reaction was complete using a ratio of at least 6.5 equivalents of the reagent to one amino group of native HEL, as shown in Table 1; derivatives of the Z group in which the aromatic ring was chlorinated could be introduced onto the amino groups of native HEL almost completely using 6.5 equivalents of the reagents to one amino group of native HEL.

Next, we examined conditions for removal of the substituents from the derivatives of HEL suitable for regenerating fully active HEL. Treatments of Boc-HEL with formic acid

for 24 h at 15°C and with HF for 90 min at 0°C gave fully active HEL in yields of 46 and 25%, respectively, and treatment of native HEL with formic acid and HF under the same conditions gave recoveries of 72 and 50%, respectively, of the active enzyme. However, enzymatically active material could not be recovered by HF-treatment of Z-HEL, prepared using Z-N₃. When native HEL was treated without Z-N₃ under similar conditions to those used for Z-N₃ treatment in preparation of Z-HEL, enzymatically active HEL was recovered in good yield and it was indistinguishable from the original enzyme in gel-chromatographic and ion-exchange chromatographic behaviors, and enzymatic activity. This suggests that some side-reactions occur during introduction of the Z group onto native HEL using Z-N₃, and that these reactions are not reversed by HF-treatment. Therefore, we next tested to regenerate enzymatically active HEL from Z-HEL, prepared by treatment of native HEL with Z-ONSu. When Z-HEL was treated with HF at 0°C for 0 and 30 min, the regenerated free amino groups were estimated as 78 and 100% of those of native HEL, respectively. These results indicate that Z groups could be removed from Z-HEL as easily as from oligopeptides by HF-treatment. HELs, substituted with Z-groups chlorinated at position(s) 2, 3, 4, 2 and 4,3 and 4, and 2 and 6 of the benzene ring, were treated with HF. The free amino groups of the original enzyme could be regenerated almost quantitatively with HF, by 30 min-treatment of Z(2-Cl) , Z(4-Cl)- and Z(2,4-Cl₂)-HELs and by 60 min-treat-

ment of Z(3-Cl)-, Z(3,4-Cl₂)- and Z(2,6-Cl₂)-HELs. Thus the relative stabilities of these groups on HF-treatment are proportional to their relative stabilities on acidolysis in 50% trifluoroacetic acid in methylene chloride reported by Merrifield et al.⁷⁾ The results show that the free amino groups of HEL can be regenerated almost quantitatively from all the derivatives of HEL examined by HF-treatment for up to 60 min at 0°C.

Next, we tried to recover fully active HEL by HF-treatment of derivatives of HEL. We found that on HF-treatment for 30 min at 0°C fully active HEL could be recovered in 41% yield from Z-HEL, in which the amino groups had been acylated almost completely under condition C in Table 1. However, the recovery decreased on increasing the period of HF-treatment. These findings are in accordance with results on HF-treatment of native HEL, described in part I. When Z-HELs prepared by the reaction of native HEL with Z-ONSu under conditions D, E and F in Table 1 were treated with HF for 30 min at 0°C, the recoveries of fully active HELs decreased in order; namely with increase in the ratio of equivalents of the reagent, Z-ONSu, to amino groups of native HEL, the recovery of fully active HEL decreased. Since fully active HEL could be recovered in 60% yield from native HEL on HF-treatment under the same conditions, the results suggest that the acylation of native HEL with Z-ONSu did not cause an irreversible change in the molecule of native HEL, but caused some side-reactions

of the molecule or that HF-treatment of Z-HEL caused some modification of the HEL molecule. Therefore, it is essential to use only the minimal amount of the reagent necessary to achieve complete acylation of the amino groups of native HEL.

In studies on the recovery of HEL from Z(X)-HELs by HF-treatments at 0°C for 60 and 90 min, fully active HEL was recovered from Z(2-Cl)- and Z(4-Cl)-HELs in approximately the same yield as from Z-HEL, but the yields from other Z(X)-HELs were lower, as shown in Table 4. These results seem inconsistent with the fact that all the Z(X) groups could be completely removed from Z(X)-HELs under the same conditions, as described already, because if this is so fully active HEL should be recovered in the same yield from all the derivatives of HEL examined. This inconsistency remains to be explained. It may be due to various extent of side-reactions during preparation of Z(X)-HELs and their treatment with HF.

We also investigated the reductive alkylations of native HEL and Z(2-Cl)-HEL and the regeneration of enzymatically active HEL from reduced and alkylated enzyme by HF-treatment. Meienhofer et al.⁵⁾ reported that native HEL can be reduced by dithiothreitol in liquid ammonia, and that the resulting thiol groups can be almost completely alkylated by benzyl chloride. Accordingly native HEL and Z(2-Cl)-HEL were reduced and alkylated by benzyl, 4-methoxybenzyl and 4-methylbenzyl chlorides by their method. Amino acid analyses of acid hydrolysates

of the products showed that native HEL and Z(2-Cl)-HEL were reduced and alkylated almost completely by benzyl groups, but partially by 4-methoxybenzyl and 4-methylbenzyl groups, as seen in Table 5. When these derivatives of HEL were treated with HF to regenerate enzymatically active HEL, it was found that the S-benzyl substituents were too stable to be cleaved from HEL(S-Bzl) under the mild conditions used for peptide synthesis, as presented in Table 6. Moreover, HEL would be destroyed under the stronger conditions necessary for complete cleavage of the S-benzyl group from HEL(S-Bzl), as described in Part I. Thus, fully active HEL could not be recovered from the HEL(S-Bzl) by HF-treatment, as shown in Table 7. It seemed probable that the S-4-methoxybenzyl substituent should be removed from HEL(S-Bzl(OMe)) and Z(2-Cl)-HEL(S-Bzl(OMe)) under appropriate conditions for HF-treatment, because it is known that the S-4-methoxybenzyl group can be almost completely removed from oligopeptides containing the S-4-methoxybenzylcysteine residue(s) by HF-treatment for 60 min at 0°C, and completely removed by treatment for 90 min at 0°C. However, only traces of fully active HEL could be recovered from either HEL(S-Bzl(OMe)) or Z(2-Cl)-HEL(S-Bzl(OMe)) by HF-treatment for 90 min at 0°C, and no fully active enzyme could be recovered by treatment for 60 min at 0°C. These low recoveries of fully active enzyme seemed to be due to use of unsuitable reaction conditions for preparing HEL(S-Bzl(OMe)) and Z(2-Cl)-HEL(S-Bzl(OMe)), because these derivatives of HEL

were always obtained as pale yellow materials. Next, HEL (S-Bzl(Me)) and Z(2-Cl)-HEL(S-Bzl(Me)) were treated with HF at 0°C for 60 and 90 min, because Erickson and Merrifield⁸⁾ reported that the S-4-methylbenzyl group like the S-4-methoxybenzyl group of S-4-methoxybenzylcysteine can be completely removed from S-4-methylbenzylcysteine by HF-treatment at 0°C for 60 min and we observed that trace amounts of S-4-methylbenzyl groups remain in S-4-methylbenzylcysteine after treatment with HF for 60 min at 0°C, whereas the groups are completely removed by HF-treatment for 90 min at 0°C. Fully active HEL was recovered in 19 and 12% yield from HEL(S-Bzl(Me)) by HF-treatment at 0°C for 60 and 90 min, respectively. Since the results seen in Table 5 indicate that about 11% of the thiol groups remained unalkylated in the HEL(S-Bzl(Me)) preparation, assuming that the reduced form of HEL constituted 11% of the HEL(S-Bzl(Me)) preparation, and that no active enzyme could be recovered from completely alkylated HEL(S-Bzl(Me)), we calculated that fully active HEL could be recovered in only 3-5% yield from the HEL(S-Bzl(Me)) preparation by HF-treatment for 60 min at 0°C. Therefore, the 19% yield of fully active HEL was probably mainly derived from the HEL(S-Bzl(Me)) preparation. Next, Z(2-Cl)-HEL(S-Bzl(Me)) was treated with HF at 0°C; fully active enzyme was recovered in yields of 8.3 and 7.4% after treatment with HF for 60 and 90 min, respectively. On the other hand, fully active HEL could be recovered in 20% yield from the reduced form of Z(2-

Cl)-HEL, in which not all the thiol groups were alkylated, by HF-treatment for 60 min at 0°C. These findings indicate that fully active enzyme was mainly obtained by cleavage of Z(2-Cl) and Bzl(Me) groups from Z(2-Cl)-HEL(S-Bzl(Me)) with HF, although 8% of the thiol groups remained to be alkylated in the Z(2-Cl)-HEL(S-Bzl(Me)) preparation.

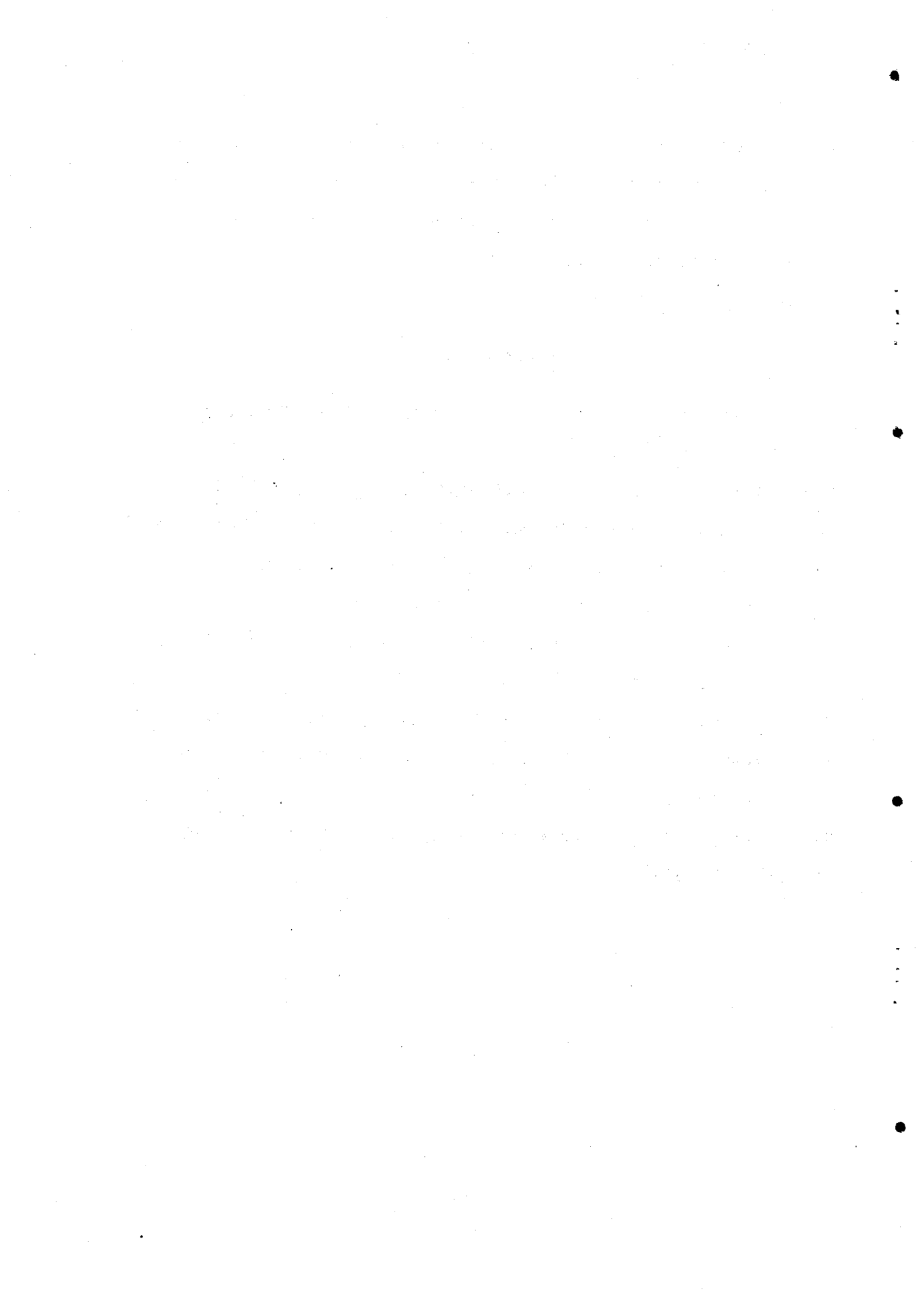
The fully active HELs recovered by HF-treatment of Z(2-Cl)-HEL and Z(2-Cl)-HEL(S-Bzl(Me)) were purified by chromatography on a Bio-rex 70 column. The main fraction, eluted in the position of native HEL, had the same activity on Micrococcus lysodeikticus cells as native HEL also purified on Bio-rex 70. The fractions eluted before the main fraction had similar activity to native HEL in buffer containing 0.1% sodium chloride, but had very low enzymatic activity in buffer containing 1% sodium chloride. They may represent deaminated derivatives of HEL formed during the chemical treatments. The main fraction eluted from the Bio-rex 70 column had the same UV-spectra as native HEL, as shown in Figs. 5 and 8. Furthermore, when the protein in this fraction was treated under appropriate conditions, it gave crystals that were identical with those of native HEL, as shown in Fig. 9.

As a further step in the chemical synthesis of hen egg-white lysozyme we prepared Z(2-Cl)-HEL(S-Bzl(Me)) as a model since it closely resembles the protected polypeptide covering the whole sequence of the lysozyme molecule required to synthesize the whole molecule. The present work showing that

fully active HEL can be regenerated in fair yield and in a crystalline state from Z(2-Cl)-HEL(S-Bzl(Me)) by treatment with HF is an encouraging indication that chemical synthesis of this enzyme should be possible.

References

- 1) S.Moore, D.H.Spackman, and W.H.Stein, *Anal.Chem.*, 30, 1185 (1958).
- 2) S.Moore and W.H.Stein, *J.Biol.Chem.*, 176, 367 (1948).
- 3) V.P.Saxena and D.B.Wetlaufer, *Biochemistry*, 9, 5015 (1970).
- 4) S.Sakakibara, Y.Shimonishi, Y.Kishida, M.Okada, and H. Sugihara, *Bull.Chem.Soc.Jpn.*, 40, 2164 (1967).
- 5) J.Meienhofer, J.Czombos, and H.Maeda, *J.Amer.Chem.Soc.*, 93, 3080 (1971).
- 6) J.Berthou and P.Jolles, *FEBS Lett.*, 31, 189 (1973).
- 7) B.W.Erickson and R.B.Merrifield, *J.Amer.Chem.Soc.*, 95, 3757 (1973).
- 8) B.W.Erickson and R.B.Merrifield, *J.Amer.Chem.Soc.*, 95, 3750 (1973).



Summary

The present work has been undertaken as the framework for the chemical synthesis of protein. This work is consisted of the following studies.

1) The influence fo anhydrous hydrogen fluoride on the native form of hen egg-white lysozyme under standard conditions of peptide synthesis were investigated by measuring the enzymatic activities of materials recovered from mixtures of anhydrous hydrogen fluoride and hen egg-white lysozyme. The enzymatic activity of hen egg-white lysozyme gradually decreased on incubation with anhydrous hydrogen fluoride at a given temperature. After incubation of the mixture at 0° C for 60 min, at least three fractions could be separated by gel-filtration on Sephadex G-50. One of these was eluted in the same position as native lysozyme and was fully active enzymatically. Its ultra-violet, and circular dichroism absorption spectra were identical with those of native lysozyme, and the crystals obtained from this fraction could not be distinguished from those of native lysozyme.

2) The influences of anhydrous hydrogen fluoride on reduced hen egg-white lysozyme were investigated under standard conditions for peptide synthesis. i) Enzymatically fully active protein was recovered in poor yield (5%) from reduced

hen egg-white lysozyme after treatment with hydrogen fluoride.

ii) The yield (29-33%) was improved by addition of 1,4-butanedithiol or L-cysteine during the treatment of the reduced hen egg-white lysozyme with hydrogen fluoride. iii) The crystals which were indistinguishable from those of native hen egg-white lysozyme were recovered after treatment of reduced hen egg-white lysozyme with hydrogen fluoride in the presence of 1,4-butanedithiol, anisole and amino acid derivatives. Thus, the original intact lysozyme could be recovered from an HF-solution of the reduced form.

3) The following derivatives of hen egg-white lysozyme were prepared from native hen egg-white lysozyme, 2-chlorobenzoyloxycarbonyl-lysozyme, S-4-methylbenzyl-reduced-lysozyme and 2-chlorobenzoyloxycarbonyl-S-4-methylbenzyl-reduced-lysozyme. Treatment of these derivatives with anhydrous liquid hydrogen fluoride for 90 min at 0°C yielded fully active lysozyme with recoveries, in order, of 23%, 12% and 7.4%. The properties of the lysozyme recovered were identical with those of native hen egg-white lysozyme, and crystals of this material were indistinguishable from those of native enzyme.

List of Publications

1. Influence of Anhydrous Hydrogen Fluoride on Hen Egg-White Lysozyme. I. Effects on Native Hen Egg-White Lysozyme
Saburo Aimoto and Yasutsugu Shimonishi
Bull.Chem.Soc.Jpn., 48, (11) 3293 (1975).
2. Influence of Anhydrous Hydrogen Fluoride on Hen Egg-White Lysozyme. II. Effects on Reduced Hen Egg-White Lysozyme
Saburo Aimoto and Yasutsugu Shimonishi
Bull.Chem.Soc.Jpn., 49, (1) 317 (1976).
3. Regeneration to the Native Form of Hen Egg-White Lysozyme from its Protected Derivatives
Saburo Aimoto and Yasutsugu Shimonishi
Bull.Chem.Soc.Jpn., to be submitted.
4. Crystallization of Synthetic Polypeptides with Triplehelical Structure
Shumpei Sakakibara, Yasuo Kishida, and Saburo Aimoto
Chemistry and Biochemistry of Peptides. Proceeding of the 3rd American Peptide Symposium p293
5. Dimerization of the Tryptophyl Moiety
Yukako Omori, Yasuhiro Matsuda, Saburo Aimoto, Yasutsugu Shimonishi, and Masao Yamamoto
Chem.Lett., 805 (1976).