# Influence of Anhydrous Hydrogen Fluoride on Hen Egg-White Lysozyme. I. Effects on Native Hen Egg-White Lysozyme

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The influences of 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 absorptions were identical with those of native lysozyme, and the tetragonal crystals obtained from this fraction could not be distinguished from those of native lysozyme.

The authors are now studying the chemical synthesis of the native form of the well-characterized protein molecule, hen egg-white lysozyme (HEL). At present they are interested in establishing how much of the intact structure of this enzyme is retained after exposure to anhydrous liquid hydrogen fluoride (HF) under usual conditions for peptide synthesis and how much of the original enzyme can be recovered in an intact form after this treatment.

HF was first used in peptide synthesis by Sakakibara *et al.*[1,2] and since then it has been widely used for releasing free peptide, because it does not show any troublesome side reactions with simple protected peptides. However, in the synthesis of complicated polypeptides, it is uncertain whether it can be used to release free polypeptides from protected derivatives without causing side reactions. HF was originally used in studies on the chemistry of peptides and proteins by Katz[3] and Katz *et al.*[3,4] examined its effects on some natural polyepptide hormones, such as insulin and corticotropin, and also on enzymes such as ribonuclease and lysozyme. They reported that the biological activities of these compounds were not irreversibly affected by HF, provided that the temperature of exposure was low (−78 °C) and the exposure time was short (<2 hr). This suggests that a considerable amount of starting material might remain in an intact form during treatment with HF under these conditions. On the other hand, they found that considerable inactivation occurred on exposure to HF at a higher temperature (0—25 °C) and for a longer time (≥2 hr), particularly in the cases of ribonuclease and lysozyme. These findings indicate that highly oriented macromolecules may be considerably affected by prolonged exposure to HF at high temperature. However, unfortunately protecting groups can only be removed from protected complicated polypeptides under these conditions. Since the syntheses of complicated polypeptide hormones and enzymes have recently become of major interest, it will be very valuable to examine the influence of HF on complicated polypeptides or enzymes under conditions generally used for peptide synthesis.

In the present study, the influences of HF on the native form of HEL were investigated in the preliminary studies of the chemical synthesis of HEL. First, the effect of the conditions of HF-treatment on the enzymatic activity of HEL was examined. Second, the attempts were made to isolate intact HEL after treatments of native HEL with HF. The results show the possibility to recover intact HEL from native HEL treated with HF under the conditions generally used for peptide synthesis.

## 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 purification. It was dissolved in dilute acetic acid, lyophilized and then dried over $P_2O_5$ *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.[2] HF (5 ml) was distilled into a cylinder containing HEL previously cooled to −78 °C in a dry-ice/methanol bath. The resulting solution was stirred at 0 °C or 25 °C for the required period. Under these conditions, the start of the reaction period was taken as 30 min after transferring the sample from the bath at −78 °C to that at 0 °C or 25 °C. 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,[5] 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 (3×60 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 dis-

solved in a buffer containing 1M acetic acid and 5M urea and charged on a column of Sephadex G-50 (fine, 2.2×95 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 (1.9×54 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. $[\theta]_R$ represents the molecular ellipticity based on the mean residue weight. Protein concentration was calculated using the value, $E^{1\%}_{1cm}=23.7$ 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 Jolles[6] with some modifications. The lyophilized protein (35 mg) was dissolved in distilled water (0.50 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 biologically 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 of 60 to 240 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.

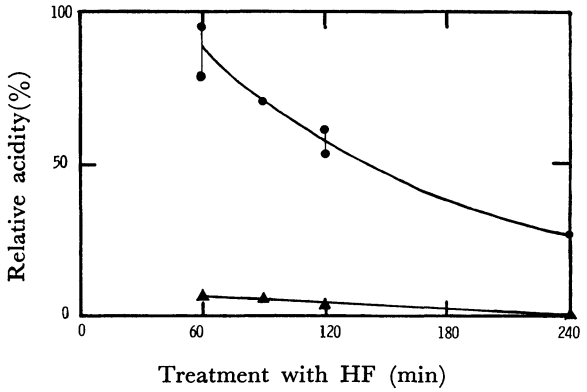


Fig. 1. Relative activity of HF-treated native HEL. The procedure used for treatment of HEL with HF and the method used of enzymatic assay of HF-treated HEL are given in the text.
—●—: treated at 0 °C, —▲—: treated at 25 °C.

The enzymatic activity of the solution of the freeze-dried native HEL treated with HF at 0 °C for 60 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 60 min its activity was only 10% of that of standard HEL and after 240 min-treatment it was almost inactive. These results are summarized in Fig. 1.

*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 60 min is shown in Fig. 2. These fractions (I, II and III) were separated. The first fraction (I) was eluted in the void voulme, and the

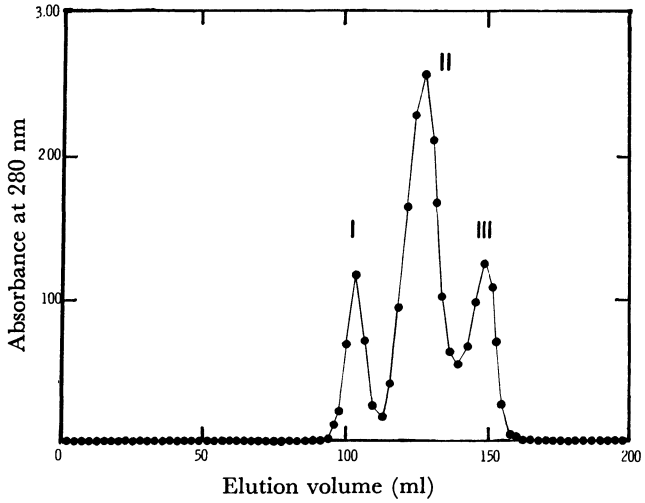


Fig. 2. Gel-filtration of native HEL treated with HF at 0 °C for 60 min on Sephadex G-50 (2.2×95 cm) using buffer containing 1 M acetic acid and 5 M urea. The flow rate was 10 ml per hr.

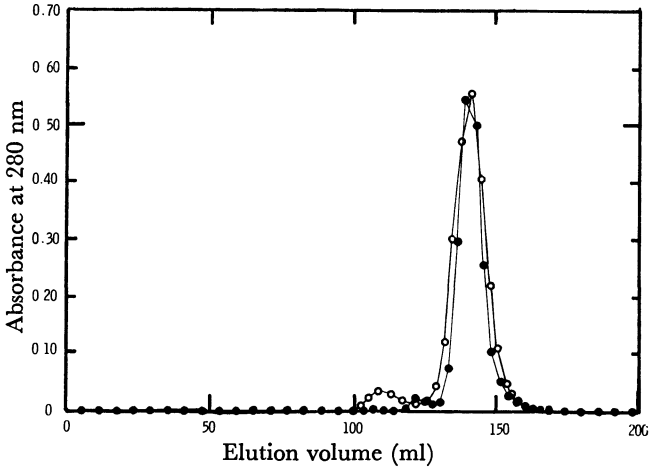


Fig. 3. Rechromatogram of fraction III of Fig. 2 on Sephadex G-50 (2.2×95 cm) using buffer containing 1 M acetic acid and 5 M urea. The flow rate was 10 ml per hr. —●—: fraction III of Fig. 2, —○—: native HEL.

third fraction (III) in the same position as standard native HEL under the same chromatographic conditions. Fraction III was rechromatographed on Sephadex G-50 using buffer containing 1M acetic and 5M urea as shown in Fig. 3. 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 the 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. 4 and 5.

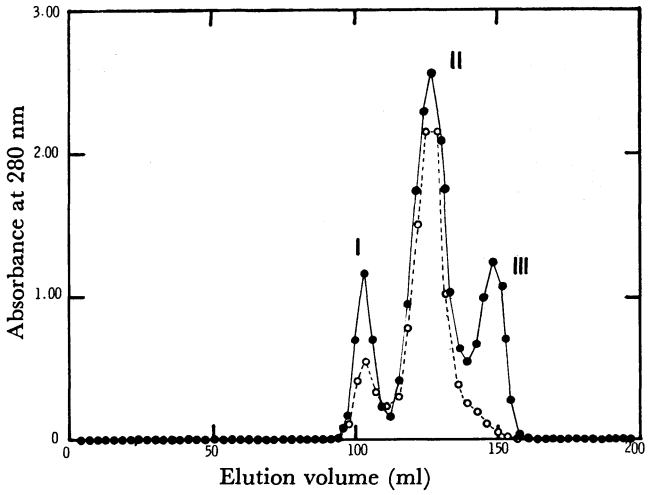


Fig. 4. Gel-filtration of native HEL treated with HF at 0 °C for 60 min (—●—) or 240 min (—○—) on Sephadex G-50 (2.2×95 cm) using buffer containing 1 M acetic acid and 5 M urea. The flow rate was 10 ml per hr.

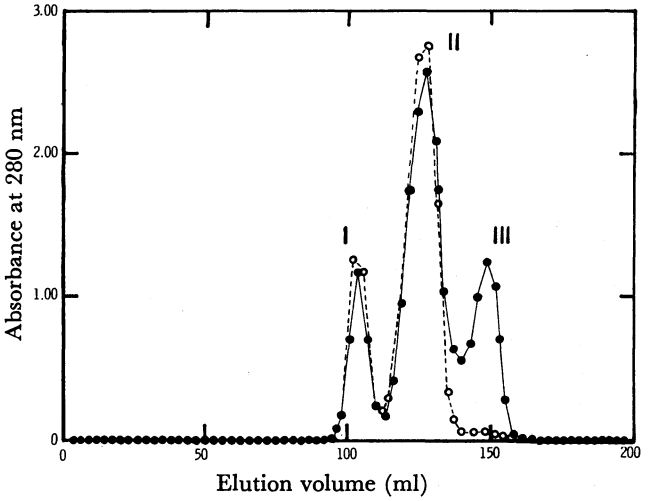


Fig. 5. Gel-filtration of native HFL treated with HF for 60 min at 0 °C (—●—) or 25 °C (—○—) on Sephadex G-50 (2.2×95 cm) using buffer containing 1 M acetic acid and 5 M urea. The flow rate was 10 ml per hr.

*Effects of Various Buffers on the Recovery of Fraction III.* The freeze-dried native HEL was treated with HF at 0 °C for 60 min and then stood 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 60 min and then chromatographed on Sephadex G-50. The fraction III obtained was subjected to chromatography on Biorex 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. 6.

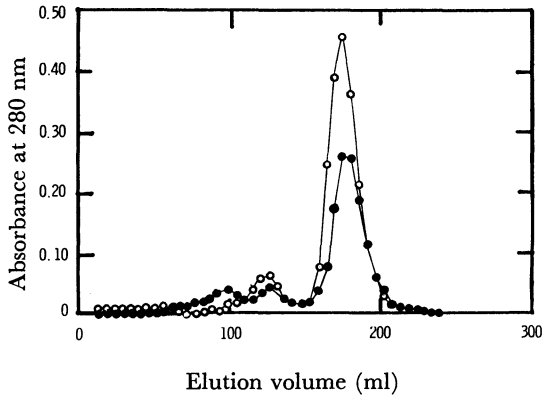


Fig. 6. Ion-exchange chromatogram of HEL on Biorex 70 (1.9×54 cm) using 0.2 M sodium phosphate buffer solution (pH 7.15). The flow rate was 16 ml per hr. —●—: fraction III of Fig. 2, —○—: native HEL.

*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-chromatographes, were determined relative to that of standard HEL on *Micrococcus lysodeikticus* cell walls. Table 2 shows the

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

| HF-treatment | Fraction I | Fraction II | Fraction III |
|---|---|---|---|
| 0 °C, 60 min | 0% | 45—66% | 97—106% |
| 25 °C, 60 min | 0 | 10—15 | — |

specific activities of fractions I, II and III, separated by gel-filtration on Sephadex G-50. Fraction III consistently showed full specific activity after treatment with HF at 0 °C for 60, 90, 120 or 240 min. The main fraction separated by chromatography of fraction III on Bio-rex 70 (Fig. 6) 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 specific activities.

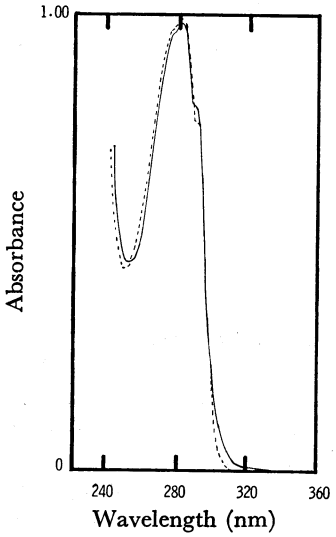


Fig. 7. Ultra-violet absorption spectra of fraction II of Fig. 2 (——) and native HEL (----). Solvent: Buffer containing 1 M acetic acid and 5 M urea.

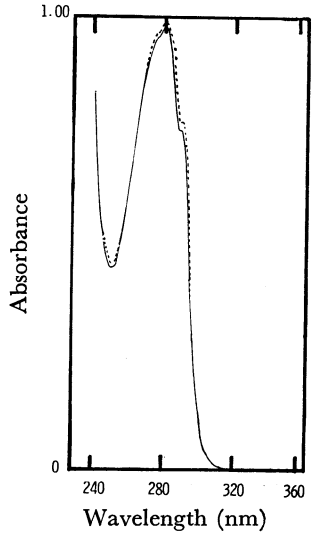


Fig. 8. Ultra-violet absorption spectra of fraction III of Fig. 2 (——) and native HEL (----). Solvent: Buffer containing 1 M acetic acid and 5 M urea.

*Absorption Spectra.* Figures 7 and 8 show the ultraviolet absorption spectra of fractions II and III, respectively in Fig. 2. 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. 9 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.

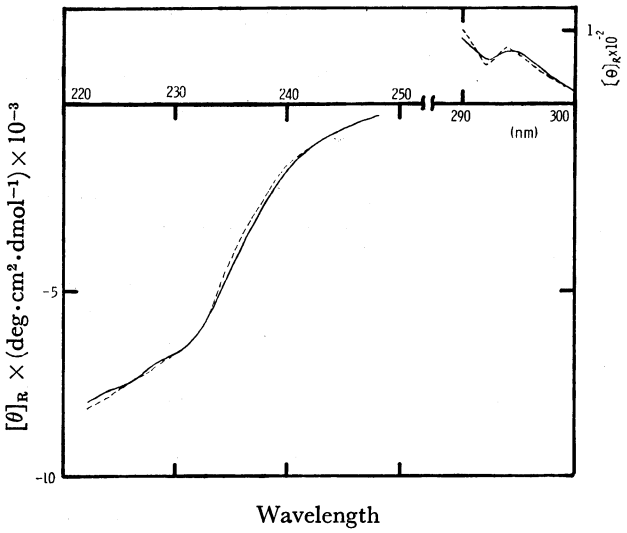


Fig. 9. Circular dichroism spectra of fraction III of Fig. 2 (——) and native HEL (----). Solvent: 0.1 M sodium chloride buffer solution adjusted to pH 3.0 with hydrochloric acid.

*Crystallization of Fraction III.* The material, recovered from fraction III in Fig. 2 was stood under conditions inducing crystallization. The tetragonal crystals obtained were like those of native HEL, as shown in Fig. 10.

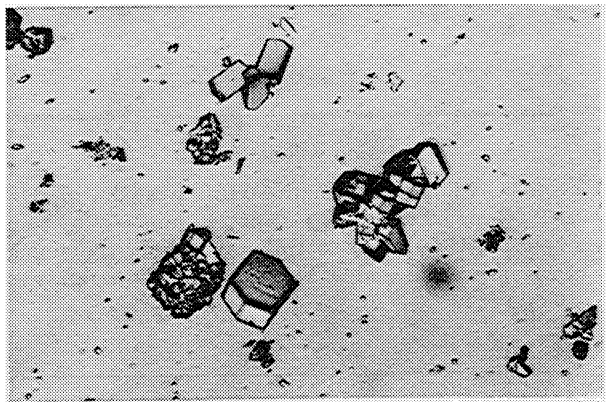

Fig. 10. Microphotograph of crystals of the material from fraction III obtained by the method of Berthou and Jolles.[7]

## 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 material 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. On gel-filtration fraction II was eluted before native HEL. The fraction II, separated after treatment of native HEL with HF at 0 °C for 60 min, had 45—66% of the activity of native HEL. However, preparations obtained after different conditions of treatment 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.[7] After treatment of native HEL with HF at 0 °C for 60 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.[7] Furthermore, when the material in fraction II was reduced and refolded under the method of Saxena and Wetlaufer,[8] 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 modification 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 conditions 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 usual conditions of peptide synthesis. The authors are now attempting to recover intact HEL from the reduced HEL treated with HF and refolded.[9] These studies should demonstrate the usefulness of HF in chemical synthesis of HEL.

## References

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