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

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The following derivatives of hen egg-white lysozyme were prepared from native hen egg-white lysozyme, 2-chlorobenzyloxycarbonyl-lysozyme, *S*-(4-methylbenzyl)-reduced-lysozyme, and 2-chlorobenzyloxycarbonyl-*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.

Previously we[1,2] reported the effect of treatment of hen egg-white lysozyme (HEL) with anhydrous liquid hydrogen fluoride (HF) under the usual conditions for peptide synthesis. We 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 paper 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-$N_3$), benzyl 2,5-dioxo-1-pyrrolidinyl 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 mercapto groups of reduced HEL. These 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-chlorobenzyloxycarbonyl and 4-methylbenzyl substituents are extremely suitable for protecting amino and mercapto 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 × 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 6 M 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.*[3] The abbreviations used in this text are those recommended by IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972).

*Synthesis of Benzyl 2,5-Dioxo-1-pyrrolidinyl Carbonate(Z-ONSu).* Benzyloxycarbonyl 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 (64.3%); 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-Dioxo-1-pyrrolidinyl 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 phosgene (25 g). The solution was kept for 20 h at room temperature in a closed vessel. The excess phosgene 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-chlorobenzyloxycarbonyl 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 the similar way to that of the benzyl derivative. 2-Chlorobenzyl 2,5-dioxo-1-pyrrolidinyl 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-Dioxo-1-pyrrolidinyl 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-Dioxo-1-pyrrolidinyl 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-Dioxo-1-pyrrolidinyl Carbonate* [*Z(2,4-$Cl_2$)-ONSu*]: Mp 92—93 °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-Dioxo-1-pyrrolidinyl Carbonate* [*Z(3,4-$Cl_2$)-ONSu*]: Mp 118—119 °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-Dioxo-1-pyrrolidinyl Carbonate* [*Z(2,6-$Cl_2$)-ONSu*]: Mp 127—128 °C. Found: C, 45.29; 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-$N_3$) (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 pentaoxide *in vacuo*. This material is denoted as Boc-HEL.

*Reaction of Native HEL with Benzyl 2,5-Dioxo-1-pyrrolidinyl Carbonate and Its Derivatives.* Native HEL was allowed to react with benzyl 2,5-dioxo-1-pyrrolidinyl carbonate (Z-ONSu) or its derivatives [Z(X)-ONSu] under the same conditions as those used with Boc-$N_3$. 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 Stein[4] using ninhydrin solution. The reagent was prepared in the following way: tin(II) chloride (0.8 g) was dissolved in 0.2 M citrate buffer (500 ml) at pH 5.0. The solution was mixed with a solution of ninhydrin (20 g) in methyl cellosolve (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:

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

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 0.2 M tris (3 ml) at pH 8.6, 5% EDTA·2Na (0.3 ml), urea (3.61 g) and 2-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 (3×60 cm). The eluate containing the protein was added to 0.08 M tris buffer solution at pH 8 (1 liter) containing $5.4\times10^{-3}$ M L-cysteine and $4.8\times10^{-4}$ M L-cystine.[5] 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 (2×30 cm, carboxylic acid cycle). The adsorbed protein was eluted with 0.3 M sodium phosphate buffer at pH 8.0. The eluate containing the protein was acidified with glacial acetic acid and lypohilized. The lyophilized material was desalted by passage through a column of Sephadex G-10 (3.2×60 cm) in 1 M 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.[6] 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 rapidly evaporated off as completely 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.*[7] 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 1 M acetic acid and 5 M urea, and chromatographed on Sephadex G-75 (3×60 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*-(carboxy-

methyl)cysteine measured as follows: Lyophilized powder (*ca.* 15 mg) of the proteins was dissolved in buffer solution consisting of 0.2 M 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 0.2 M 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 iodoacetic acid (270 mg) in 1 M 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 6 M hydrochloric acid, and the ratios of the amount of *S*-(carboxymethyl)cysteine 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 paper.[1] 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 paper,[1] with native HEL as a standard.

*Ultraviolet 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 Jolles[8] with the modification described in the preceding paper.[1]

## Results

*Reaction of Native HEL with Boc-$N_3$.* When native HEL was treated with Boc-$N_3$ 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-$N_3$ repeatedly under the same conditions, 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.

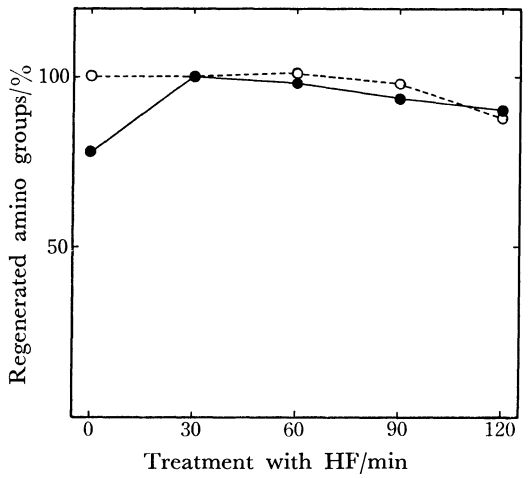


Fig. 1. Removal of Z groups from Z-HEL by HF-treatment.
—●—: Z-HEL, —○—: native HEL.

Table 3. Percentages 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 |

*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.

*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 conditions 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 1 M acetic acid and 5 M 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.

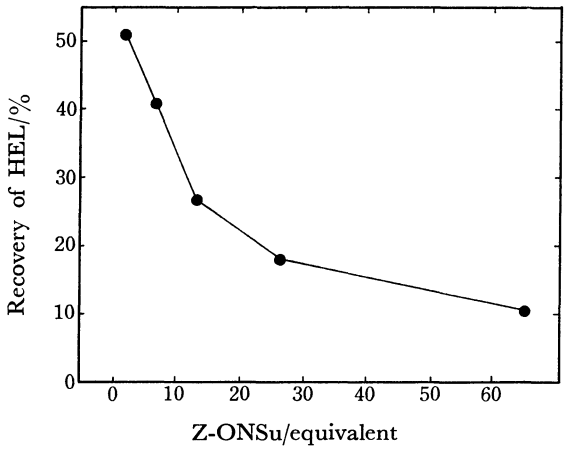


Fig. 2. Effect of the ratio of equivalent of Z-ONSu to amino groups of native HEL used in the synthesis of Z-HEL on the recovery of fully active HEL from Z-HEL by HF-treatment.

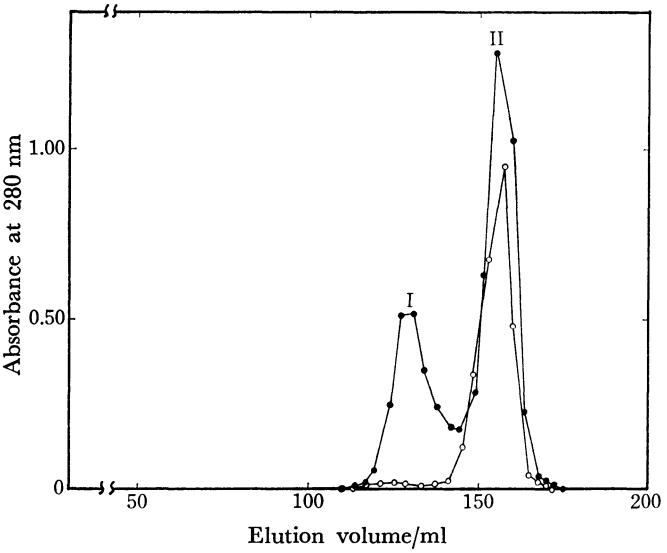


Fig. 3. Gel-filtration of Z(2-Cl)-HEL treated with HF for 60 min at 0 °C (—●—) and native HEL (—○—) on Sephadex G-50 (2×95 cm) using buffer containing 1 M acetic acid and 5 M urea.

*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, 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 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 0.2 M sodium phosphate buffer solution, pH 7.18, as shown in Fig. 4. In this way four fractions (IIa, IIb, IIc, and IId) were separated. Fraction IId was eluted in the same position as the main fraction of native HEL. The lytic activities of the four fractions, IIa, IIb, IIc, and IId, 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 solution containing 1% sodium chloride. The UV absorption spectrum of fraction IId is shown in Fig. 5. Fraction

Table 4. Recoveries of fully active HEL from Z(X)-HELs by HF-treatment

| Conditions for HF-treatment | Native HEL | Z-HEL | Z(X)-HEL | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 2-Cl | 3-Cl | 4-Cl | 2,4-$Cl_2$ | 3,4-$Cl_2$ | 2,6-$Cl_2$ |
| 0 °C, 60 min | 59 | 28 | 22 | 10 | 20 | 16 | 7.3 | 16 |
| 0 °C, 90 min | 50 | 23 | 23 | 18 | 26 | 14 | 9.5 | 16 |

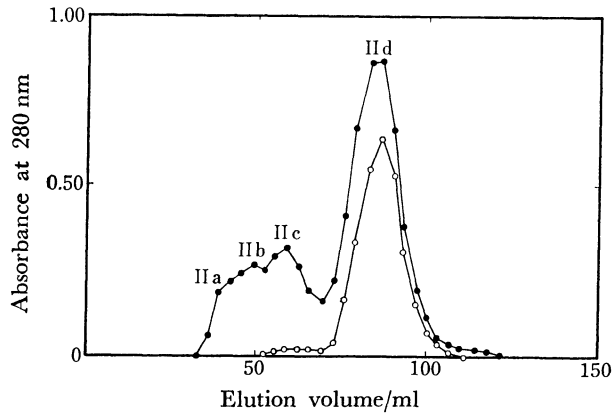


Fig. 4. Ion-exchange chromatogram of fractionII of Fig. 3 (—●—) and native HEL(—○—) on Bio-rex 70 (1.5×33 cm) in 0.2 M sodium phosphate buffer solution (pH 7.18).

IId was desalted by passage through Sephadex G-10, and the eluate containing protein was collected and lyophilized. When the 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 Ammomia.* 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, determined by amino acid analysis of the acid hydrolysate of the alkylated product, as shown in Table 5. It was difficult to estimate the degrees of alkylation with 4-methoxybenzyl and 4-methylbenzyl groups by measuring *S*-(4-methoxybenzyl)cysteine and *S*-(4-methylbenzyl)cysteine in acid hydrolysates of the alkylated products by amino acid analysis, because these *S*-(4-methoxybenzyl)- and *S*-(4-methylbenzyl)cysteine 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*-(carboxymethyl)cysteine 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 6 M 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.

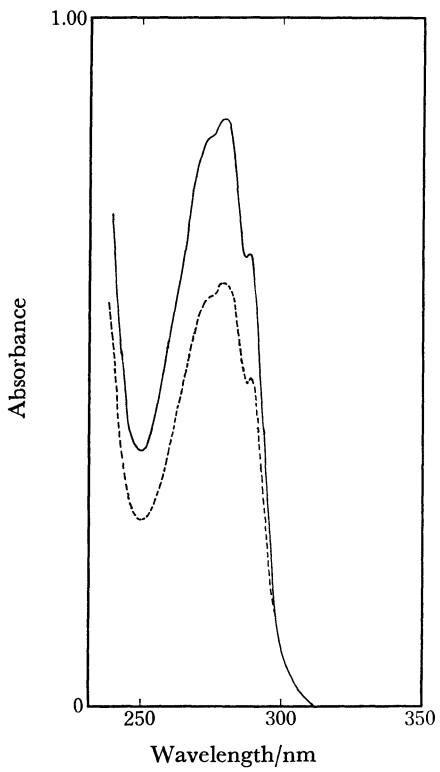


Fig. 5. Ultraviolet absorption spectra of fraction IId of Fig. 4 (——) and native HEL (----). Solvent: 0.2 M sodium phosphate buffer (pH 7.18).

*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 7.

Table 5. Reductive alkylations of native HEL and Z(2-Cl)-HEL

| Product | Starting material | (mg) | (μmol) | Liq.$NH_3$ (liter) | DTT[a] (mmol) | Alkylating reagent | (mmol) | Alkylated SH groups (residue mol) |
|---|---|---|---|---|---|---|---|---|
| HEL(*S*-Bzl) | Native HEL | 1000 | 67 | 2.0 | 5.3 | Bzl-Cl | 26.4 | 8.16 |
| HEL(*S*-Bzl(OMe)) | Native HEL | 300 | 20 | 2.0 | 2.0 | Bzl(OMe)-Cl | 20.0 | 6.80(85.0%)[b] |
| HEL(*S*-Bzl(Me)) | Native HEL | 200 | 13 | 1.0 | 1.1 | Bzl(Me)-Cl | 11.0 | 7.12(89.0%)[b] |
| Z(2-Cl)-HEL(*S*-Bzl(OMe)) | Z(2-Cl)-HEL | 120 | 8 | 1.0 | 1.0 | Bzl(OMe)-Cl | 10.0 | 7.36(92.0%)[b] |
| Z(2-Cl)-HEL(*S*-Bzl(Me)) | Z(2-Cl)-HEL | 240 | 16 | 1.8 | 2.0 | Bzl(Me)-Cl | 19.0 | 7.36(92.0%)[b] |

a) DTT: Dithiothreitol. b) Values in parentheses show the ratios of the amount of alkylated mercapto groups to the theoretical value (100%).

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——15 °C | 4 | 2 | 5 |
| 0 °C | 20 | 32 | 35 |

Table 7. 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 1 M acetic acid and 5 M urea, as shown in Fig. 6. 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 7. Lyophilized material was obtained from fraction II by desalting and chromatographed on a Bio-rex 70 column using 0.2 M sodium phosphate buffer solution at pH 7.18. Fraction II was separated into four fractions

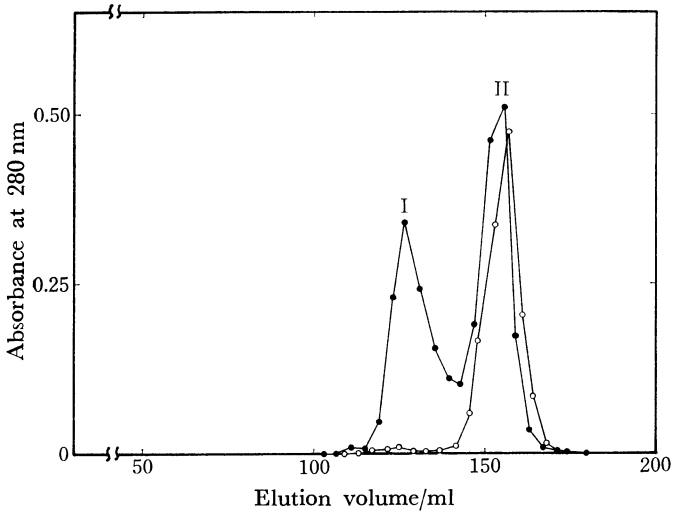


Fig. 6. Gel-filtration of Z(2-Cl)-HEL(*S*-Bzl(Me)) treated with HF for 60 min at 0 °C (—●—) and native HEL (—○—) on Sephadex G-50 (2×95 cm) in buffer containing 1 M acetic acid and 5 M urea.

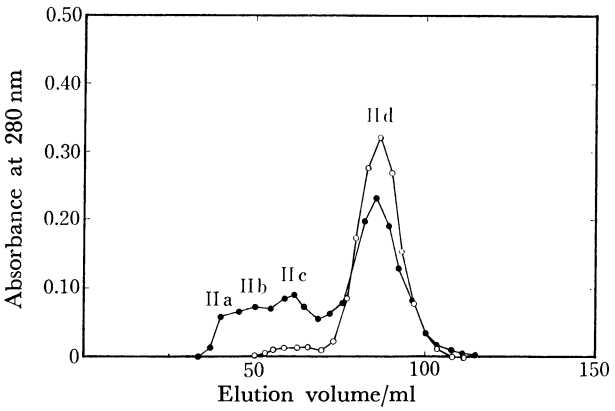


Fig. 7. Ion-exchange chromatogram of fraction II of Fig. 6 (—●—) and native HEL (—○—) on Bio-rex 70 (1.5×33 cm) in 0.2 M sodium phosphate buffer solution (pH 7.18).

(IIa, IIb, IIc, and IId), as shown in Fig. 7. Fraction IId was eluted in the same position as the main fraction of native HEL. These four fractions, IIa, IIb, IIc, and IId, 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 IId is shown in Fig. 8. When the lyophilized powder obtained from fraction IId (Fig. 7) after desalting was stood under conditions inducing crystallization, it yielded similar crystals to those of native HEL, as shown in Fig. 9.

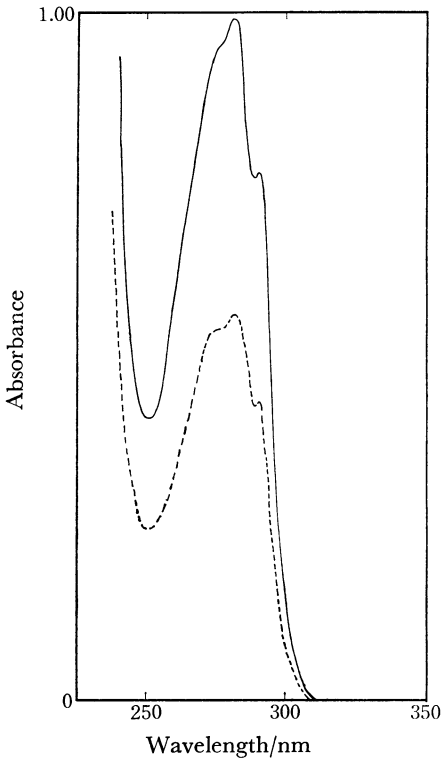


Fig. 8. Ultraviolet absorption spectra of fraction IId of Fig. 7 (——) and native HEL (----). Solvent: 0.2 M sodium phosphate buffer (pH 7.18).

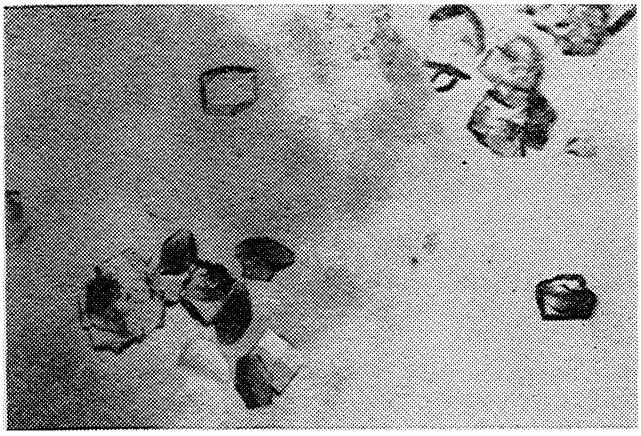

Fig. 9. Microphotograph of crystals of the material from fraction IId of Fig. 7.

## 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 were prepared by treating the enzyme with Boc-$N_3$ or Z-ONSu or derivatives of the latter [Z(X)-ONSu]. The first reagent was not very reactive so that repeated treatment was required to achieve complete acylation of the amino groups of native HEL. Then, we tested Z-ONSu, which can easily be prepared by the reaction of benzyloxycarbonyl chloride (Z-Cl) with *N*-hydroxysuccinimide (HONSu), and found that 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 to regenerate enzymatically active HEL from the derivatives of 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. Namely, the recovery of fully active HEL from Boc-HEL was about one half of that from native HEL. These results suggest that some side-reactions occur during the treatment of Boc-HEL with formic acid or HF, or that some side-reactions occur during introduction of the Boc group onto native HEL using Boc-$N_3$ and are not reversed by formic acid- or HF-treatment, whereas these reactions were not examined in this work. However, these results show that enzymatically active material can be recovered in fair yields from Boc-HEL under the conditions described above. We next tested to regenerate enzymatically active HEL from Z-HEL and Z(X)-HELs. We first determined free amino groups regenerated by the treatment of Z-HEL and Z(X)-HELs, in which the amino groups are completely protected, with HF under various conditions. 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. Then, HELs, substituted with monochloro- or dichloro-Z group, 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-$Cl_2$)-HELs and by 60 min-treatment of Z(3-Cl)-, Z(3,4-$Cl_2$)- and Z(2,6-$Cl_2$)-HELs. Thus the relative stabilities of these groups on HF-treatment are proportional to their relative stabilities on acidolysis in 50% trifluoracetic acid, in methylene chloride reported by Merrifield *et al.*[9] The results show that the free amino groups of HEL can be regenerated quantitatively from all the derivatives of HEL examined by HF-treatment for up to 60 min at 0 °C. Then, we tried to isolate fully active HEL from the products obtained 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 conditions 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 the preceding paper[1]. 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 69% 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. It has been described[9] that use of Z(2-Cl) group for $\varepsilon$-amino protection of lysine reduces branching at the amino acid residue in peptide synthesis, whereas Z and Z(4-Cl) groups are sufficiently labile in acidic conditions that peptide chains undergo branching at the side chain of lysine residue during peptide synthesis if either group is used for $\varepsilon$-amino protection. This and our results suggest the usefulness of Z(2-Cl) group for $\varepsilon$-amino protection of lysine residue in our studies on the chemical synthesis of HEL. Therefore, HEL substituted with Z(2-Cl) groups was examined further in detail. On the other hand, the recoveries of HEL from other Z(X)-HELs were lowered, 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 extents of side-reactions during preparation of Z(X)-HELs and their treatments 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 enzymes by HF-treatment. Meienhofer *et al.*[7] reported that native HEL can be reduced by dithiothreitol in liquid ammonia, and that the resulting mercapto 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 the preceding paper.[1] Thus, fully active HEL could not be recovered from the HEL(*S*-Bzl) by HF-treatment. 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-methoxybenzyl)cysteine 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. Erickson and Merrifield[10] reported that *S*-4-methylbenzyl group can be completely removed from *S*-(4-methylbenzyl)cysteine by HF-treatment at 0 °C for 60 min. However, we observed that trace amounts of the group remain in *S*-(4-methylbenzyl)cysteine after treatment with HF under the same conditions, whereas the group is completely removed by HF-treatment for 90 min at 0 °C. Therefore, we treated the above materials with HF at 0 °C for 60 and 90 min. 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. Table 5 shows that about 89% of the mercapto groups were alkylated in the HEL(*S*-Bzl(Me)) preparation; namely, about 11% of the mercapto groups remained unalkylated. Assuming that 11% of the mercapto groups were exclusively constituted by the reduced form of HEL, and that no active enzyme could be recovered from completely alkylated HEL(*S*-Bzl(Me)), we can deduce 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, from simple calculation ($0.11 \times 0.33 \times 100\% = 3.63\%$), since the reduced form of HEL gave fully active HEL with 33% yield under the same conditions.[2] Therefore, the 19% yield of fully active HEL was probably mainly derived from *S*-4-methylbenzylated HEL. 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 mercapto 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 mercapto 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.

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