Insulin Aggregation in Aqueous Media and Its Effect on Alpha-Chymotrypsin-Mediated Proteolytic Degradation

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Self-association of zinc–insulin monomers into dimers and hexamers may lead to enhanced protection of the peptide from proteolytic degradation. The present study has been undertaken to investigate the relationship, if any, between the rate of enzymatic degradation of insulin by a protease, alpha-chymotrypsin, and the extent of insulin aggregation in aqueous solutions. Insulin solutions (0.6 mg/ml) containing varying proportions of dimer and hexamer were obtained by adding ethylene diamine tetraacetic acid (EDTA) within a concentration range of 0.005 to 0.040 m*M*. As the EDTA concentration was increased above 0.040 m*M*, a complete dissociation of hexamers to dimers occurred and the rate of enzymatic degradation reached its maximum. The overall first-order rate constants appeared to be linearly related to the square of EDTA concentrations. The apparent first-order rate constants for dimer and hexamer degradation obtained from a linear plot of rate constant versus EDTA squared concentration were found to be 0.02800 ± 0.00065 and 0.00798 ± 0.00075 min^{-1}, respectively. Two major insulin degradation products were also detected and the kinetics of product appearance agreed well with the disappearance kinetics of insulin. The results indicated that the degradation of insulin dimers by alpha-chymotrypsin is about 3.5 times faster than the degradation of the hexamer. The second-order dependency of degradation rate on EDTA concentration might be due to the fact that insulin hexamers contain two zinc ions which are sequestered by two EDTA molecules. Chelation of zinc ions by EDTA lead to hexamer deaggregation to dimers as was evidenced from a circular dichroism study. Formation of three dimer species from one hexamer aggregate should theoretically enhance the rate of degradation threefold, a value consistent with the experimentally determined ratio of 3.5.

KEY WORDS: alpha-chymotrypsin; insulin degradation; insulin dimer and hexamer; EDTA effect; circular dichroism.

INTRODUCTION

Zinc insulin can be viewed as a long-acting dosage form of insulin due to its slow absorption and low biodegradation at the site of administration. Insulin molecules in the presence of zinc ions in aqueous solution tend to undergo self-association to form primarily dimers and hexamers (1). Different aggregated forms of insulin may exhibit different absorption rates *in vivo* (2). Studies of the effect of zinc ions on insulin metabolism by liver plasma membranes indicated that zinc ions can enhance insulin receptor binding and concomitantly decrease insulin metabolism (3). Conversely, minimization of insulin aggregation in aqueous solution is useful in developing the long-term polymer-coated insulin delivery system (4,5). EDTA can prevent insulin self-association in aqueous solutions by sequestering zinc ions from insulin molecules (6).

Our primary interest in studying alpha-chymotrypsin-mediated degradation of insulin stems from results obtained during investigation of intestinal mucosal transport and metabolism of insulin with the goal of development of oral insulin delivery systems (7). It has been observed that alpha-chymotrypsin is the major gastrointestinal proteolytic enzyme responsible for initial cleavage and unfolding of insulin globular structure, exposing the molecule to subsequent attack by brush border and enterocytic enzymes. Insulin diffusion coefficients depend to a large extent on the molecular weight of the associated species. The smaller the aggregation number of insulin, the higher will be its permeation rate across a mucosal epithelial barrier (oral route) or capillary endothelial barrier (subcutaneous route) (8). However, smaller species (dimers) will have higher degradation rates than larger species (hexamers) because for the same unit mass, smaller species will offer a correspondingly larger number of exposed peptide linkages susceptible to proteolytic enzyme attack. Therefore, a balance must be created between optimum rate and extent of absorption and biodegradability of peptides and proteins at the site of administration.

This report describes a quantitative study on the *in vitro* degradation of porcine zinc insulin in the presence of alpha-chymotrypsin, a model enzyme selected for its high proteolytic activity. Insulin solutions containing varying fractions of the dimers and hexamers have been obtained by adding EDTA; the hexamer/dimer interconversion has been studied using circular dichroism (CD).

MATERIALS AND METHODS

Materials

Crystalline porcine sodium and zinc insulin were gifts from Eli Lilly and Company (Indianapolis, IN). Lyophilized alpha-chymotrypsin prepared from bovine pancreas (56 units/mg protein) was purchased from Sigma Chemical Company, St. Louis, MO. Acetonitrile and deionized water (both HPLC grade) were obtained from Baxter Healthcare Corporation (Muskegon, MI). Phosphoric acid and triethylamine were obtained from Fisher Scientific (Fairlawn, NJ). Trifluoroacetic acid (TFA), EDTA, and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Sigma Chemical and were of HPLC, ACS reagent, and reagent grades, respectively. Deionized double-distilled water was used throughout the study.

HPLC Assay of Insulin

The insulin assay was performed on a computer-controlled gradient high-pressure liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet visible de-

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tector (Knauer, West Germany). The gradient system used in this study consisted of mobile phase A, triethylammonium phosphate (TEAP) solution prepared by adjusting the pH of 0.25 *N* phosphoric acid to 2.25 with triethylamine, and mobile phase B, 100% acetonitrile. The gradient system was programmed by increasing the proportion of mobile phase B from 24 to 40% in mobile phase A within 40 min. Twenty microliters of the sample was injected onto a Rainin reversed-phase C-8 Microsorb column (250 × 4.6 mm) connected to a C-8 precolumn. The gradient mobile phase was run at a flow rate of 1 ml/min. The ultraviolet/visible detector was set at 220 nm; the recorded signal was analyzed with an electronic integrator (Model 3390A, Hewlett-Packard Co., Avondale, PA). The chromatographic method is specific for insulin and suffers from no interference by insulin metabolites or alpha-chymotrypsin.

Incubation of Insulin Solutions with Alpha-Chymotrypsin

Insulin stock solution (2.0 mg/ml) was prepared by accurately dissolving 50 mg of crystalline porcine zinc insulin in 25 ml of 0.1 *M* Tris–HCl buffer (pH 7.4). Alpha-chymotrypsin in Tris–HCl solution (0.125 mg/ml) was used as the incubation medium. An aliquot (0.3 ml) of insulin stock solution was pipetted into six 1.5-ml microcentrifuge tubes (DOT Scientific Inc., Flint, MI). Several aliquots (0, 0.05, 0.1, 0.2, 0.3, and 0.4 ml) of 0.1 *M* Tris–HCl buffer (pH 7.4) containing 0.1 m*M* EDTA were then added to these tubes. The volume of each sample was adjusted to 0.9 ml with Tris–HCl buffer. Tubes were placed in a 37°C shaker water bath (Haake, West Germany). An aliquot (0.1 ml) of alpha-chymotrypsin solution preequilibrated at 37°C was accurately pipetted into each tube, and the mixture was vortexed immediately. The final EDTA concentrations in these incubation mixtures were 0, 0.005, 0.01, 0.02, 0.03, and 0.04 m*M*, respectively. Using similar dilution procedures, insulin solutions containing higher EDTA concentrations (0.05, 0.1, 0.2, and 0.4 m*M*) were also prepared. Samples (0.1 ml) were withdrawn from the incubation mixture at 0, 10, 20, 30, and 40 min postincubation, immediately mixed with 0.2 ml of 5% TFA solution, and subsequently stored in a freezer at −20°C until HPLC analyses were performed. Studies were performed in triplicate.

Insulin Degradation Kinetics and Effects of EDTA

Scheme I describes the pathways of insulin degradation in a zinc insulin/alpha-chymotrypsin mixture containing various EDTA concentrations, where dimers and hexamers are presumably the two dominant species in solution. An assumption must be made regarding rapid equilibration between hexamers and dimers following EDTA addition.

The rate constants k_h and k_d denote the apparent first-order rate constants associated with hexamer and dimer degradation, respectively. Therefore, the rate of total loss of insulin in the system can be expressed by Eq. (1).

$$-\frac{d[I]_t}{dt} = k_d \cdot f_d[I]_t + k_h \cdot f_h[I]_t = k_{obs} \cdot [I]_t \quad (1)$$

where k_{obs} is the experimentally observed apparent first-order rate constant, $[I]_t$ is the total insulin concentration, and f_d and f_h are the corresponding fractions for insulin dimers and hexamers. Then Eqs. (2) and (3) are obtained:

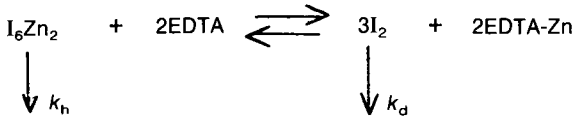
$$k_{obs} = k_d \cdot f_d + k_h \cdot f_h \quad (2)$$

$$f_d + f_h = 1 \quad (3)$$

Combining Eqs. (2) and (3), Eq. (4) can be obtained:

$$k_{obs} = (k_d - k_h) \cdot f_d + k_h \quad (4)$$

Since f_d varies with EDTA concentration, k_{obs} is a function of EDTA concentration. Therefore, k_d and k_h can be calculated from a plot of k_{obs} against f_d.



Scheme I. Insulin interaction and degradation pathways.

Circular Dichroism Study

An insulin (both sodium and zinc) concentration of 1.0 mg/ml has been used throughout this study, because at or above this concentration, CD clearly differentiates dimer and hexamer spectra and illustrates progressive transition from one species to the other. A CD spectropolarimeter (JASCO Model J600, Japan Spectoscopic Co., Tokyo) was utilized to illustrate the fractional changes in insulin dimer and hexamer concentration following the addition of EDTA. Zinc insulin solutions containing various concentrations (0.04, 0.08, and 0.16 m*M*) of EDTA were scanned from 400 to 240 nm at a scanning rate of 50 nm/min and at a temperature of 22°C because distinctive and nonoverlapping changes in insulin CD spectra could be obtained with these EDTA concentrations. The CD spectra of sodium insulin solutions containing 0 and 0.16 m*M* EDTA (the highest concentration of EDTA used in the zinc insulin study) were obtained under the same conditions. Specific ellipticities, $[\psi]_\lambda = \theta_\lambda/(C \cdot l)$ (where θ_λ is the observed ellipticity at a given wavelength λ, C is the insulin concentration as grams per milliliter, and l is the pathlength as decimeters), were calculated for the entire wavelength range mentioned above.

RESULTS AND DISCUSSION

Insulin and its metabolites can be separated by HPLC (Fig. 1). The peak areas of insulin (III) and two major metabolites (I and II) were measured. The metabolite structures have been discussed in a previous report (7). Insulin biodegradation by alpha-chymotrypsin is characterized as a first-order process. As shown in Fig. 2, the rate of reaction is dependent on the EDTA concentration. The apparent first-order rate constants of insulin degradation by alpha-chymotrypsin were calculated from the slope of the semilogarithmic plot of insulin concentration remaining as a function of time. An increase in degradation rate with an increase in EDTA concentration is probably due to the dissociation of zinc insulin hexameric aggregates. The rate of appearance of insulin initial metabolites (I and II) also increases with EDTA concentration (Figs. 3A and B). The addition of

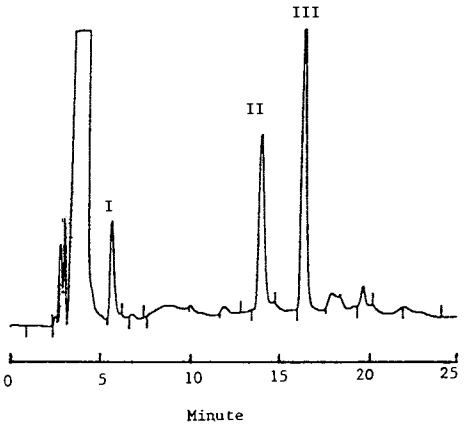


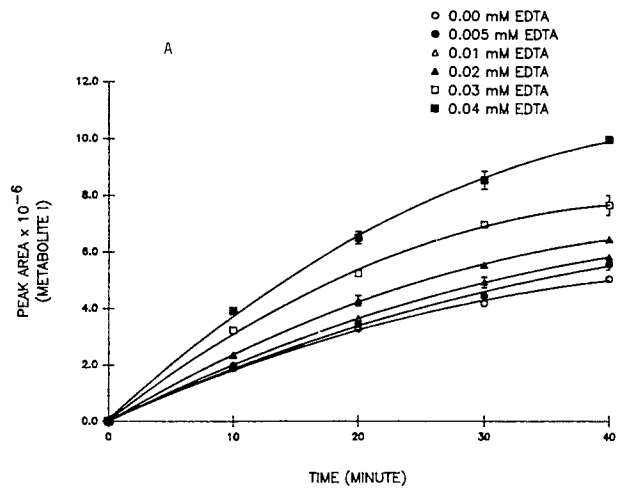
Fig. 1. HPLC diagram of the sample taken from the insulin/alpha-chymotrypsin mixture, where peaks I and II refer to initial insulin metabolites generated by the enzyme, and peak III represents intact insulin.

EDTA to insulin aqueous solutions tends to cause the dissociation of zinc insulin hexamers to form dimers due to the chelation of zinc ions. The fraction of insulin dimers in the system consequently increases with EDTA concentration and, eventually, might reach a steady state.

The results of enzymatic degradation showed that the change of the observed apparent first-order rate constant is proportional to the square of total EDTA concentration over a range of 0 to 0.04 mM (Fig. 4), i.e.,

$$k_{obs} = m[\text{EDTA}]^2 + c \qquad (5)$$

where m and c are constants. In this case, calculated values for m and c are 7.094 min^{-1} $\text{m}M^{-2}$ and 0.008052 min^{-1}, respectively. The observed rate constant, k_{obs}, reaches its plateau at an EDTA concentration of 0.05 mM or above (Fig. 5), where a steady state has been reached relative to zinc ion chelation. At that point, it is reasonable to assume that insulin dimers are the dominant species, i.e., $f_d = 1$ and $k_d = k_{obs}$. The rate constant for hexamer degradation, k_h, is calculated by assuming that the solution without EDTA consists mainly of the hexamers. The values for k_d and k_h in terms of loss of insulin monomers from dimeric and hexameric species are 0.0280 ± 0.00065 and 0.00798 ± 0.00075 min^{-1} (±SD; n = 3), respectively. Further, from Eqs. (4) and (5), Eq. (6) is obtained.



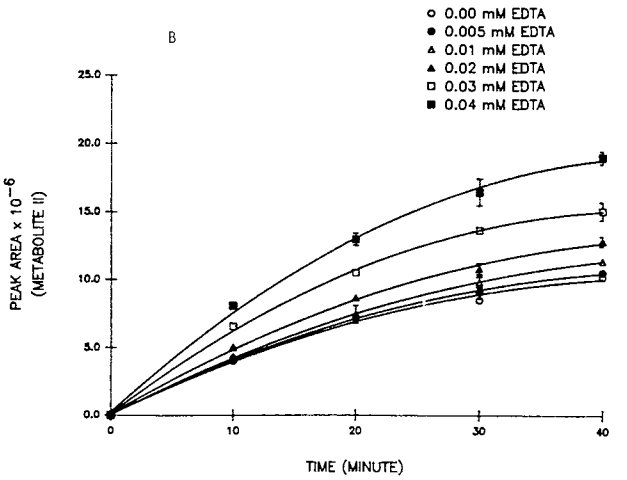


Fig. 3. The effect of various EDTA concentrations on the formation of insulin initial metabolite I (A) and metabolite II (B).

$$f_d = \frac{m}{(k_d - k_h)} \cdot [\text{EDTA}]^2 \qquad (6)$$

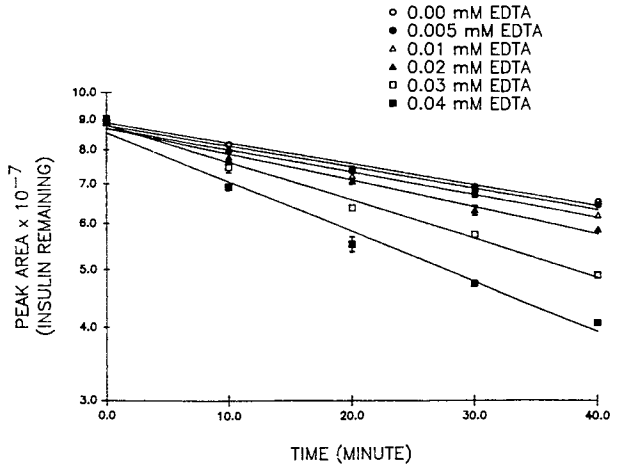


Fig. 2. Semilogarithmic plot of insulin biodegradation by alpha-chymotrypsin in the presence of EDTA.

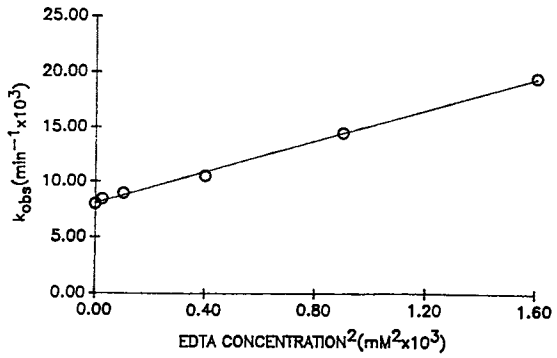


Fig. 4. The relationship between experimental apparent first-order rate constants of insulin degradation by alpha-chymotrypsin and various EDTA concentrations.

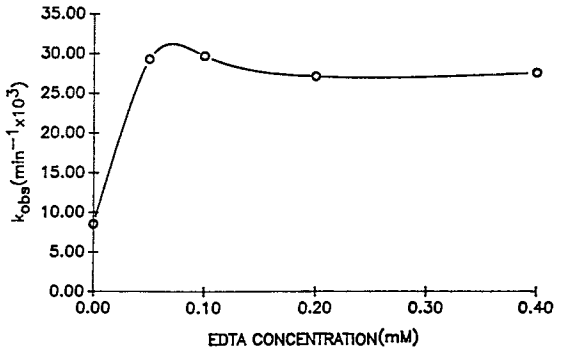


Fig. 5. The effect of EDTA concentration on the apparent first-order constants of insulin degradation.

By fitting Eq. (6) to the experimental data, Eq. (7) has been obtained; this quantitatively describes the relationship between EDTA concentration (m*M*) and dimer fraction over a EDTA concentration range of 0 to 0.04 m*M*.

$$f_d = 623\ [\text{EDTA}]^2 \qquad (7)$$

The fraction of insulin dimers generated by EDTA is proportional to the square of the EDTA concentration. The second-order dependency on EDTA could be due to the fact that a zinc insulin hexamer contains two zinc ions, which are sequestered by two EDTA molecules. The observed apparent first-order constants and the calculated insulin dimer fractions are summarized in Table I. The rate of dimer degradation is about three and half times higher than that of hexamer. The differences in insulin hexamer and dimer susceptibility to enzymatic reaction can be explained by collision theory. Because an insulin hexamer dissociates into three dimers, a complete dissociation of insulin hexamer may increase the number of insulin species three times. The dissociation of insulin hexamer may cause the apparent first-order rate to increase by about threefold. This prediction turns out to be accurate from our experimental data, which shows a 3.5-fold stabilization. The presence of EDTA does not alter the bioactivity of alpha-chymotrypsin as evidenced by an incubation study of sodium insulin with alpha-chymotrypsin. The degradation rate of sodium insulin in the presence of 0.16 m*M* EDTA (0.02583 ± 0.00134 min^{-1}) is not significantly different from that in the absence of EDTA (0.02626 ± 0.00121 min^{-1}). This suggests that EDTA does not change the conformation of insulin dimers and the bioactivity of alpha-chymotrypsin.

Self-association of insulin in aqueous media and the effect of EDTA on such insulin aggregation have been experimentally supported by results obtained using an ultraviolet CD technique capable of demonstrating the changes in dimer and hexamer fractions at a given insulin concentration (6). The CD spectra of zinc and sodium insulin solutions in the absence and presence of EDTA are presented in Figs. 6A–C. The results showed that the specific ellipicity of zinc insulin is gradually decreased with increasing EDTA concentration (Fig. 6C), whereas the CD spectra of sodium insulin showed no change in the presence of EDTA (Fig. 6B), suggesting no apparent change in insulin dimer conformation by EDTA. The CD spectra of zinc and sodium insulin solutions differ significantly (Fig. 6A). According to previous reports (6,9), the negative CD absorption in Zn-insulin at about 270 nm is contributed by phenylalanine and tyrosine aromatic residues in the B_{23-28} region of the antiparallel beta-structure present in the insulin aggregated form. Therefore, the attenuation of negative maxima at about 270 nm is due to dissociation of insulin aggregates, namely, from hexamers into dimers. Further, the CD spectrum of zinc insulin solution (1 mg/ml) became very similar to that of sodium insulin in the presence of 0.16 m*M* EDTA; this might imply that insulin hexamers were dissociating nearly completely into dimers because sodium insulin in solution exists mainly in the dimeric form.

Table I. Calculated Insulin Dimer Fraction and Apparent First-Order Rate Constants (±SD; n = 3) as a Function of EDTA Concentrations

	EDTA concentration (m*M*)					
	0.00	0.005	0.01	0.02	0.03	0.04
Dimer fraction	0.00	0.016	0.06	0.25	0.56	0.99
$k_{obs} \times 10^3$ (min^{-1})	7.979 ±0.752	8.425 ±0.370	8.936 ±0.166	10.510 ±0.582	14.436 ±1.106	19.483 ±0.610

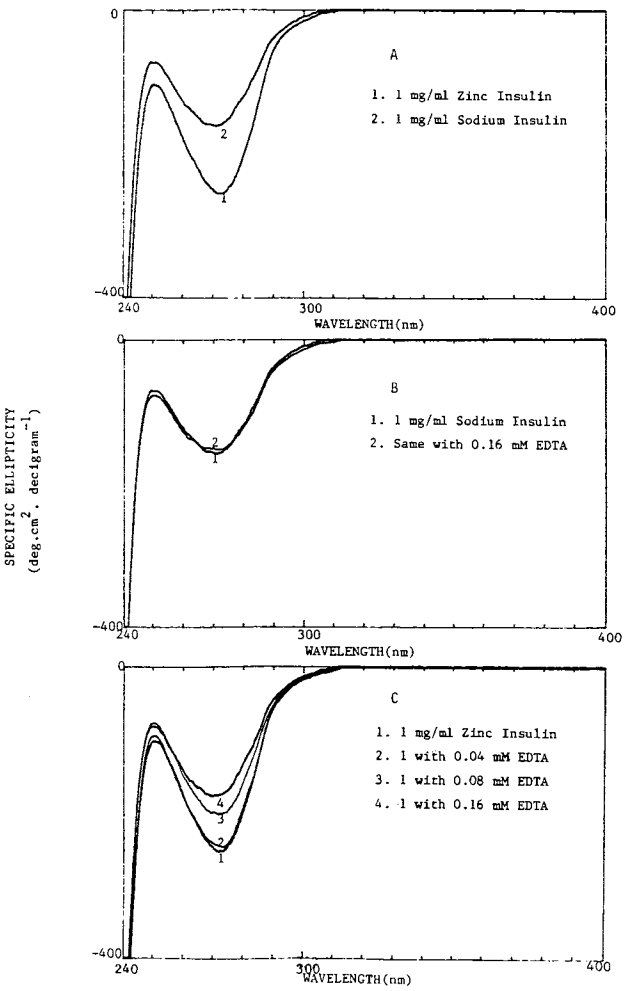


Fig. 6. The CD spectra of zinc and sodium insulin solutions containing various concentrations of EDTA.

This phenomenon confirms that the rate of change of insulin degradation by alpha-chymotrypsin is caused by an increase in insulin dimer concentration in solution.

In conclusion, the rate of biodegradation of insulin in the hexameric form is slower than in the dimeric form. The increase in the total number of insulin aggregates in solution induced by EDTA results in higher insulin-enzyme collision frequency, which in turn produces a corresponding increase in the rate of degradation. For an insulin delivery system, insulin aggregation should be prevented in order to obtain a high permeability coefficient across an absorption barrier. However, lower aggregation would enhance the susceptibility of insulin to proteolytic degradation at the absorption site. Insulin hexamers have the advantage of significantly decreasing enzymatic degradation, but their large molecular dimensions may lead to a lower permeability coefficient across the mucosal barrier. Therefore, a decision to select the proper insulin aggregate form should be made based on the objective of a specific insulin delivery system. The degradation studies presented in this report are *in vitro* in nature and one has to be careful in directly extrapolating the results to an *in vivo* situation without further study.

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