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Chemical and α-Chymotrypsin-Mediated Proteolytic Degradation of Insulin in Bile Salt-Unsaturated Fatty Acid Mixed Micellar Systems

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The proteolytic degradation of porcine zinc insulin by α-chymotrypsin was previously found to depend markedly on the state of insulin aggregation (*Pharm. Res.* 9:864–869, 1992). In this study, the effect of bile salt-unsaturated fatty acid mixed micelles on α-chymotryptic degradation of insulin was further characterized. The incorporation of linoleic acid has greatly accelerated insulin degradation with the apparent first order rate constant being linearly related to the concentration of linoleic acid. At a 10 m*M* linoleic acid concentration solubilized in 10 m*M* sodium glycocholate, the proteolytic degradation rate constant increased by 16 times, which could not be explained solely by the mechanism of insulin oligomer dissociation. Further, this effect is significantly reduced when the free carboxylic group of linoleic acid is methylated. The catalytic role of mixed micelles on chemical degradation of insulin was found to depend on the concentration of linoleic acid incorporated. When solubilized in the form of mixed micelles, linoleic acid chemically catalyzes peptide bond cleavage in a concentration-dependent manner.

KEY WORDS: α-chymotrypsin; fatty acid; insulin; mixed micelles; proteolytic degradation; stability.

INTRODUCTION

Proteins and polypeptides are degraded both enzymatically and nonenzymatically following various pathways and mechanisms (1). The mucosal transport and stability patterns of insulin have attracted much attention of formulation scientists. Insulin is prone to cleavage by a variety of luminal and brush border enzymes such as pepsin, trypsin, α-chymotrypsin, aminopeptidases, and carboxypeptidases (2–5). Degradation of insulin by various organ homogenates was also characterized systematically (6).

Nonenzymatic stability of purified crystalline insulins and their formulations has also been reported previously (7–9). Fisher and Porter (7) proposed that deterioration of crystalline bovine insulin follows a combination of two mechanisms, i.e., deamidation and polymerization. Deamidation occurs through the hydrolysis of the side-chain amide group in glutaminyl or asparaginyl residues to form a free carboxylate (10). Inspection of the insulin primary sequence reveals six such sites, i.e., Gln^{A5}, Gln^{A15}, Asn^{A18}, Asn^{A21}, Asn^{B3}, and Gln^{B4}, of which the three asparagine residues are most labile for hydrolysis (8). Relative to deamidation, insulin polymerization occurs to a lesser extent because of the higher activation energy barrier involved. The hydrolytic kinetics of insulin in hydrochloric acid was studied using HPLC method (11). The pseudo-first-order rate constant was greatly affected by the concentration of HCl and temperature, while many degradation products formed during the course of reaction.

Macromolecular aggregation and surface adsorption are two major obstacles limiting the physical stability of insulin solutions for long-term infusion. Qinn and Andrade (12) and Sato *et al.* (13) have demonstrated that incorporation of various additives such as amino acids, EDTA (ethylenediaminetetraacetic acid), Tris buffer, bicarbonate buffer, and urea could greatly improve insulin stability and aqueous solubility. This effect was further attributed to either the chelation mechanism or hindrance of the insulin–insulin interaction.

Recently, nonparenteral insulin delivery through oral, nasal, pulmonary, rectal, and even conjunctival routes has been investigated as possible alternative pathways in controlling long-term diabetes. However, difficulties are generally encountered in promoting noninvasive insulin delivery, and absorption enhancers need to be incorporated to improve insulin bioavailability. A variety of chemical enhancers, such as bile salts (14,15), fatty acids (16), bile salt–fatty acid mixed micelles (17–19), ionic and nonionic surfactants (20), and cyclodextrin derivatives (21,22), have been studied with respect to their toxicity and efficacy. Successful formulation of such complicated delivery systems will depend not only on the permeability enhancing efficacies but also on insulin stability in these formulations against both chemical and enzymatic cleavage. Our previous reports (4,23,24) focused on the study of insulin degradation by trypsin and α-chymotrypsin and the effects of certain deaggregating agents on such degradative processes. Bile salts were found to facilitate insulin oligomer dissociation through micelle formation, which led to increased proteolytic breakdown of insulin. The objective of this study was to characterize porcine zinc insulin degradation by α-chymotrypsin in the presence of bile salt-fatty acid mixed micelles. The hydrolytic stability of insulin in this mixed micellar system has also been investigated.

MATERIALS AND METHODS

Materials

Crystalline porcine zinc insulin was a gift from Eli Lilly and Company (Indianapolis, IN). Lyophilized α-chymotrypsin prepared from bovine pancreas (56 U/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Baxter Health care Corporation (Muskegon, MI). Phosphoric acid and triethylamine were obtained from Fisher Scientific (Fairlawn, NJ). Trifluoroacetic acid (TFA), sodium glycocholate (NaGC), unsaturated fatty acids, unsaturated fatty acid methyl esters, and tris(hydroxymethyl)aminomethane (Tris) were provided by Sigma Chemical Co. Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade.

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HPLC Analysis of Insulin

Insulin analysis was performed on a computer-controlled gradient high-pressure liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet-visible detector (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 26 to 40% within 20 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 and the recorded signal was analyzed with an electronic integrator (Model 3390 A, Hewlett-Packard Co., Avondale, PA). This chromatographic method provides baseline separation of insulin from its metabolites.

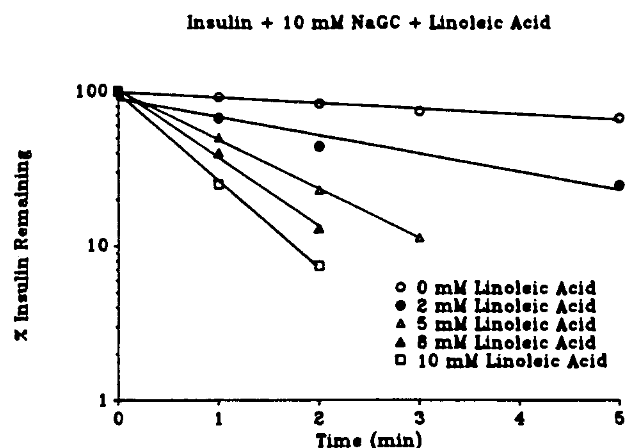

Fig. 1. Semilogarithmic plots of zinc insulin biodegradation by α-chymotrypsin in mixed micellar solutions containing 10 m*M* sodium glycocholate and varying concentrations of linoleic acid. The initial concentrations of insulin and α-chymotrypsin were 0.5 mg/mL and 0.5 μ*M*, respectively.

α-Chymotryptic Degradation of Zinc Insulin

Ten milliliters of 0.5 mg/mL zinc insulin solution was prepared in a buffer composed of 100 m*M* Tris and 1 m*M* $CaCl_2$ adjusted to pH 8.0. NaGC was then added into the solution to achieve 10 m*M* concentration and incubated at 37°C for 10 min. A fatty acid or its methyl ester was then added and sonicated for approximately 5 min just prior to taking the 0-time sample. Then 50 μL of enzyme stock solution (0.1 m*M*) was added to insulin to generate a final enzyme concentration of 0.5 μ*M* or 0.7 U/ml and the reaction initiation time was recorded. Aliquots (100 μL) were withdrawn at 1, 2, and 5 min and immediately added into 0.9 mL 0.2% TFA solution to arrest the reaction. The samples were subsequently stored in a freezer at −20°C until HPLC analyses were performed. Studies were performed in triplicate.

Chemical Instability of Zinc Insulin in Mixed Micellar Solutions

The chemical stability studies of insulin in mixed micellar solutions were also performed using similar procedures. Ten milliliters of 0.5 mg/mL zinc insulin solution was prepared in 100 m*M*, pH 7.4, Tris buffer. Sodium glycocholate (NaGC) was then added to insulin solution, which was pre-equilibrated for 10 min at 37°C. The 0-time sample was taken just prior to the addition of fatty acid. The pH of the solution was readjusted to 7.4 if necessary. Aliquots (100 μL) were then withdrawn at predetermined time intervals for up to 24 hr and immediately diluted with 0.9 mL Tris buffer. The samples were stored in a freezer for subsequent HPLC analyses. Studies were performed in triplicate.

RESULTS AND DISCUSSION

α-Chymotrypsin-Mediated Insulin Biodegradation

The degradation profiles of porcine zinc insulin by α-chymotrypsin are depicted in Fig. 1. The linear profiles can be well fitted by the apparent first-order kinetics. The first-order rate constant was found to be 0.0799 ± 0.0260 min^{-1} (mean ± SD; $n = 3$) in the absence of any fatty acid. This rate constant represents incomplete dissociation of insulin hexamers by 10 m*M* NaGC (24). When linoleic acid was incorporated in the form of mixed micelles, substantial increases in the rate and extent of insulin degradation were observed. The apparent first-order rate constants again exhibit direct dependency on the fatty acid concentration as shown in Fig. 2. It is important to note that the first-order rate constants correlate linearly with the fatty acid concentrations. Above 10 m*M*, linoleic acid could not be completely solubilized in the system and experiments were not performed beyond this concentration. Linear regression of the data points resulted in an expression in the form of $k_{obs} = 0.1200C_{LA} + 0.1324$. An increase in k_{obs} of 16.3-fold was obtained as the linoleic acid concentration was increased from 0 to 10 m*M*. This magnitude cannot be accounted for simply by insulin hexamer dissociation, in which a maximum increase of sixfold should be observed when insulin hexamers completely dissociate into monomeric species. Therefore, this observation tends to indicate a catalytic effect of linoleic acid on α-chymotrypsin-mediated insulin biodegra-

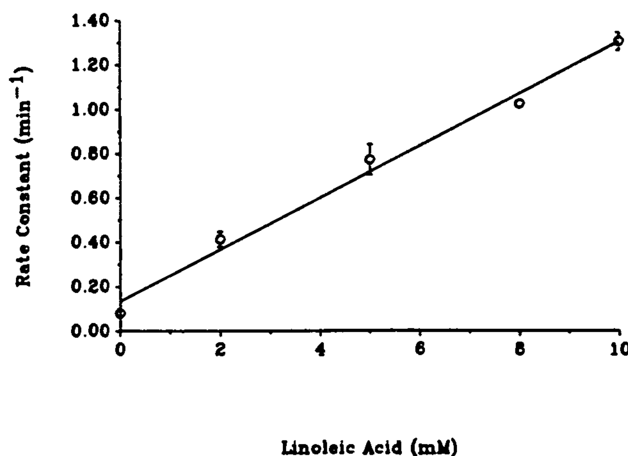

Fig. 2. Correlation between the pseudo-first-order rate constant of insulin biodegradation by α-chymotrypsin and the concentration of linoleic acid solubilized in 10 m*M* NaGC. Each value represents the mean ± SD ($n = 3$).

dation. Such catalysis could be due to the direct attack of linoleic acid on the insulin molecules, an allosteric regulation of linoleic acid on α-chymotrypsin, or a combination of both. In addition, dissociation of insulin hexamers also result in changes in hydrophobicity, thermodynamics, etc., which also plays an important role in enzyme-mediated proteolytic degradation of insulin in the presence of mixed micelles.

In order to clarify further the involvement of the lipophilic hydrocarbon chain versus the free carboxylic group of the fatty acid in the catalytic process of insulin biodegradation, methylated esters of linoleic acid and linolenic acid were examined. The degradation rate constants were once again plotted in Fig. 3 in the form of a bar chart. Apparently, linolenic acid (9,12,15-octadecatrienoic acid) exhibited a similar catalyzing effect to linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid). Furthermore, when the free carboxylic group is methylated, substantial reductions in the apparent first-order rate constants were observed. The rate constants were statistically smaller than that of the free acids ($P < 0.05$, Student's t test), indicating the importance of free carboxylic group in the fatty acid in α-chymotrypsin-mediated degradation of insulin.

Stability of Insulin in Bile Salt–Fatty Acid Mixed Micellar Solutions

Since fatty acids markedly facilitated insulin degradation, efforts were directed to finding plausible reasons for such an effect. First, incubation of insulin in mixed micelles without the presence of the proteolytic enzyme will substantiate whether a direct hydrolytic effect exists. A concentration of 30 m*M* NaGC was then used for this purpose in order to solubilize enough fatty acids. Figure 4 schematically represents the hydrolytic degradation profiles of 0.5 mg/mL zinc insulin in the presence of 0–30 m*M* linoleic acid. All lines appear to follow the apparent first-order kinetics. NaGC alone did not show any measurable effect on insulin breakdown. Incorporation of 30 m*M* linoleic acid in 30 m*M* NaGC, however, markedly promoted insulin hydrolysis to an extent that only 21.14% of intact insulin remained at the end of the 24-hr incubation at 37°C. Such an effect has never been reported in the literature previously.

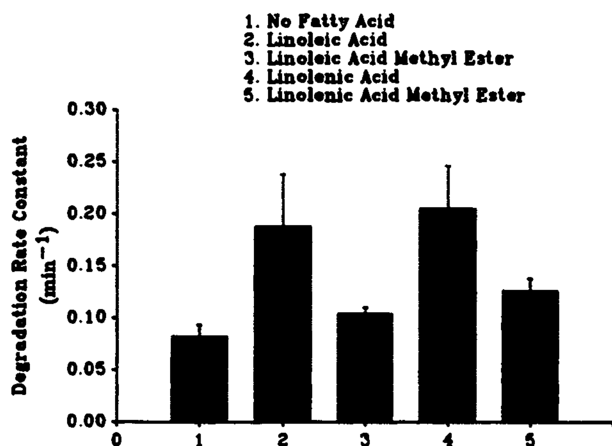

Fig. 3. Effect of unsaturated fatty acids and their methyl esters on α-chymotrypsin-mediated insulin biodegradation. The concentrations of NaGC and lipids were 10 and 2 m*M*, respectively. Bars denote means ± SD (n = 3).

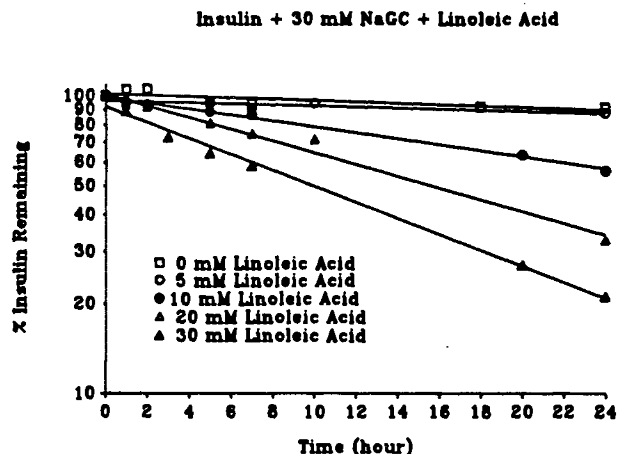

Fig. 4. Effect of varying concentrations of linoleic acid on chemical degradation of porcine zinc insulin in mixed micellar solutions. The concentration of NaGC was fixed at 30 m*M*. Values represent the average of three determinations.

Figure 5 illustrates the relationship between hydrolytic rate constants and linoleic acid concentrations. Above 5 m*M* linoleic acid, increases in k_{obs} appear to be linearly correlated with linoleic acid concentration in the mixed micellar solutions. Linear regression revealed a linoleic acid-catalyzed second-order rate constant (k_{LA}) of 2.952 $hr^{-1} \cdot M^{-1}$ (r = 0.998).

Although solubilized linoleic acid indeed catalyzes insulin hydrolysis, the underlying mechanism differs significantly from that of α-chymotrypsin-mediated insulin degradation. α-Chymotrypsin cleaves proteins and peptides rapidly and specifically at the C side of hydrophobic amino acid residues. Linoleic acid, on the other hand, appears to cleave peptide bonds slowly and randomly. Attempts to separate and isolate any degradation products using both gel filtration and electrophoresis were proven unsuccessful. HPLC gradient programming also failed to separate any major metabolite peaks. HPLC chromatograms following 0.5 mg/mL insulin storage in mixed micellar solution incubated for 24 hr also demonstrated an increase in the number of peaks.

Micellar catalysis of hydrophobic substrates by ionic surfactant molecules has been well documented (25). Micelle formation reportedly facilitates substrate hydrolysis by more than two orders of magnitude, provided the compound is

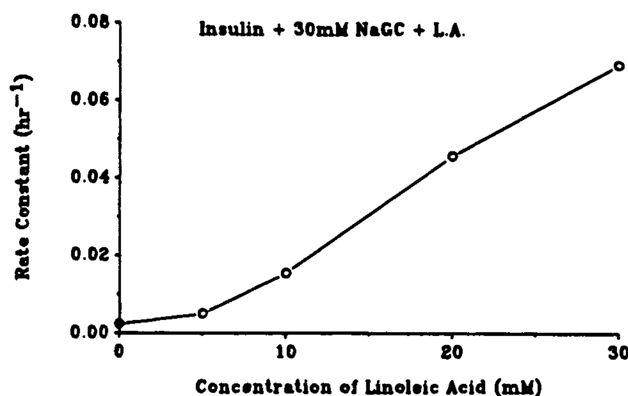

Fig. 5. Correlation between mixed micelle-catalyzed insulin degradation and linoleic acid concentration. Each value represents the average of three determinations.

efficiently bound to the micelles. Our previous kinetic study and circular dichroic spectra (24) indeed substantiated a strong interaction between insulin hexamers and bile salt micelles, leading to complete insulin hexamer dissociation. Since fatty acid molecules also actively associate with bile salts forming mixed micelles, cleavage of insulin peptide bonds by closely associated fatty acid anions is a reasonable mechanism.

Unsaturated fatty acids in solution (or in biological membranes) can contain or easily form peroxides in the presence of molecular O_2 and transient metal ions (Zn^{2+}-insulin). Peroxidation of such lipids leads to free and highly stable radicals that finally cause the breakdown (fragments) and/or nonspecific polymerization of insulin. The observation that linoleic acid (two double bonds) has less effect than linolenic acid (three double bonds) also tends to support this hypothesis.

Insulin hydrolysis by fatty acids in the micellar form may be responsible in part for insulin loss in the presence of α-chymotrypsin. However, this slow process plays only a limited role if fatty acids do not have any direct effect on the enzyme. Since the enzymatic degradation of insulin was greatly facilitated in the presence of micellar fatty acids, possible regulation of enzyme activity by fatty acid molecules is likely to be another underlying mechanism. It is well-known that the binding of fatty acids to some proteins such as albumin influences their conformation, which may have consequences for other binding sites. An increase over 10 times in urapidil-to-human serum albumin binding constant was recently reported by Storck and Kirsten (26) due to the addition of fatty acids in the system. It is not known, at present, whether fatty acids bind to enzymes like α-chymotrypsin. Should such an event occur, a possible allosteric regulation on the catalytic activity of the enzyme may arise through a strong binding efficiency between the hydrophobic binding pocket in the enzyme and aromatic or bulky residues in the insulin molecule. More work is definitely needed to clarify this hypothesis.

The importance of this finding also poses a new question on the effectiveness of orally delivered proteins and polypeptides. Aside from the concern of luminal and brushborder enzymatic cleavage, natural fatty acid content in the intestinal tract may accelerate the breakdown of peptide-like drugs to an uncontrollable extent.

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