Degradation of Insulin by Trypsin and Alpha-Chymotrypsin

Robert J. Schilling¹ and Ashim K. Mitra¹,²

Received August 20, 1990; accepted January 9, 1991

The rate and extent of insulin degradation by trypsin and α-chymotrypsin were examined in vitro, and the initial sites of cleavage by α-chymotrypsin were identified. The apparent $K_m$ for both enzymes was approximately the same but the apparent $V_{max}$ for α-chymotrypsin was 8.6 times greater. At a molar ratio of 172:1 (insulin:enzyme), chymotrypsin caused a near-total loss of insulin within 40 min, while very little insulin was degraded by trypsin. Chymotrypsin appeared to cleave initially at the carboxyl side of the B26-Tyr and A19-Tyr residues, and additional cleavage at the B16-Tyr, B25-Phe, and A14-Tyr residue sites also occurred rapidly. Only two to three other susceptible bonds, which are not exposed at the surface of the insulin molecule, remained intact after the quenching of initial cleavage. Four of the amino acids involved in initial cleavage are essential for receptor binding ability, making it difficult to modify insulin chemically to achieve greater stability without losing activity.

KEY WORDS: insulin; chymotrypsin; trypsin; degradation.

INTRODUCTION

Trypsin and α-chymotrypsin, the major proteolytic enzymes secreted by the pancreas into the intestinal lumen, are both known to cleave various bonds within the insulin molecule (1-6). In a previous paper from this laboratory (7) the apparent permeability of insulin across everted rat intestinal gut sacs was reported to be significantly greater in the mid to distal jejunum and ileum than in the duodenum, and degradation of insulin by brush border enzymes of intact gut sacs was negligible in all regions. A dosage form that would selectively deliver insulin to the jejunum could optimize its absorption and may decrease its degradation, since luminal proteolytic enzyme concentrations would be lower than in earlier portions of the small intestine. It is still expected that despite protection from enzymatic degradation, the low intrinsic permeability of insulin would require an absorption enhancer. Nevertheless, insulin must still be protected from the pancreatic digestive enzymes to provide the greatest amount of intact, biologically active insulin to be available for absorption.

Coadministration of insulin with different enzyme inhibitors, either nonspecific or specific for trypsin or chymotrypsin, has shown potential for increasing the absorption of bioactive insulin from the intestine (8-13). One group of investigators (12) reported that insulin in doses of 1.4-4.2 U/kg with a chymotrypsin inhibitor administered together orally in an enteric-coated capsule produced substantial lowering of blood glucose in nondiabetic human subjects. On the other hand, two earlier studies which delivered insulin to the duodenum of human patients lacking pancreatic digestive enzymes found that 50–100 U insulin/kg was required to lower blood glucose effectively, while control subjects showed no apparent absorption (14,15). For reference, typical insulin doses administered subcutaneously to diabetics are in the range of 0.5–1.0 U/kg/day (16). Although insulin absorption from the small intestine in the above studies may have required large doses, protecting insulin from enzymatic degradation appears to have produced significant results. Extensive administration of enzyme inhibitors to the digestive tract, however, is not a therapeutically sound principle since digestion and absorption of a meal must also occur at the same time in which blood insulin levels are elevated.

Thus, the objectives of the present research are twofold. First, it is desirable to compare the initial kinetics and the overall extent of insulin degradation by trypsin and α-chymotrypsin. Trypsin itself cleaves insulin at only two sites: on the carboxyl side of residues B29-Lys and B22-Arg (5,6) (nomenclature B29-Lys, for example, indicates lysine, the 29th residue of the B chain). The former metabolite retains insulin-like activity, but the latter (desoctapeptide-insulin) has virtually no activity (6). Since the bonds susceptible to tryptic cleavage are relatively exposed at the carboxyl terminus of the B chain (17), at least the latter bond would need to be protected to prevent inactivation of insulin. Alpha-chymotrypsin, on the other hand, can cleave at least seven bonds in insulin (14-4). Previous studies have addressed only either the formation of metabolites after a relatively long period of incubation or the end products of the enzymatic digestion. The second objective, then, is to determine whether any specific bonds within the insulin molecule are especially susceptible to initial cleavage by chymotrypsin. As described above, it is conceivable that preventing this initial cleavage (by subtly altering susceptible bonds) might also protect the entire insulin molecule from chymo-

¹ Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907.
² To whom correspondence should be addressed.
tryptic degradation. If such a scheme were possible, luminal degradation of insulin delivered to the small intestine would be minimized or prevented, thus increasing the amount of intact insulin available for absorption.

MATERIALS AND METHODS

Materials

Purified porcine zinc insulin, in crystalline form, was kindly donated by Eli Lilly and Company (Indianapolis, IN). Lyophilized type XIII TPC-trypsin from bovine pancreas, lyophilized type VII TLCK-trypsin α-chymotrypsin from bovine pancreas, benzoylarginine, and benzoylarginine-ethyl ester (BAEE) were obtained from Sigma Chemical Company (St. Louis, MO). HPLC-grade phosphoric acid, triethylamine, and trifluorocetic acid (TFA) were purchased from Fisher Scientific (Pittsburgh, PA), and Burdick and Jackson HPLC-grade water and acetonitrile (ACN) were purchased from Scientific Products (McGaw Park, IL). All other chemicals were of analytical reagent grade.

Insulin Degradation by Trypsin Versus Chymotrypsin

Before examining the enzymatic degradation of insulin, it was necessary to compare directly the activities of trypsin and chymotrypsin in units of bonds cleaved per unit time. As purchased, the activity of chymotrypsin was specified as 57 BTEE units per mg, defined as 57 μmol of benzoyltyrosine-ethyl ester (BTEE) hydrolyzed per min. The activity of trypsin was specified as 12,000 BAEE units per mg, which is defined, however, as a change in UV absorbance at 253 nm per unit time. Therefore, the assay procedures as used by Sigma Chemical Company were repeated using trypsin and BAEE, measuring the increase in absorbance at 253 nm due to the production of benzoylarginine. The absorbance of standard solutions of mixtures of BAEE and benzoylarginine were then used to calculate the number of micromoles of BAEE cleaved by trypsin per unit time.

The extent of insulin degradation by equimolar concentrations of trypsin and α-chymotrypsin was examined as follows. Ten milliliters of 17.24 μM insulin solutions was prepared in a buffer composed of 100 mM Tris and 1 mM CaCl₂ adjusted to pH 8.0. The insulin solutions were equilibrated for 15 min at 37°C. Just prior to the addition of enzyme, the insulin solution was vortexed for 2 sec and a 100-μl sample was immediately added to a measured volume of a cold solution of Tris buffer and TFA, which lowered the pH of the sample to pH 2.5. This procedure, used throughout the experiments, arrested the enzymatic degradation yet maintained insulin stability. Trypsin or chymotrypsin was dissolved in room-temperature buffer and equilibrated for a period of 12 min before use. Then 50 μl of enzyme solution was added to insulin solutions to generate a final trypsin or chymotrypsin concentration of 0.1 μM. The digest solution was vortexed for 2 sec. Periodically, 100-μl samples were taken and diluted with cold Tris/TFA buffer as described.

After refrigeration overnight, insulin concentrations were determined by a gradient elution HPLC system (Rainin Instruments, Woburn, MA) with UV detection at 220 nm (Knauer, Model 87, Berlin). All samples were assayed in random order. A reversed-phase Microsorb C-8 column, 5 μm, 4.6 × 250 mm, (Rainin Instruments), was employed with a flow rate of 1 ml/min using triethylammonium phosphate, pH 2.25 (TEAP 2.25), as mobile phase A and ACN as mobile phase B. TEAP 2.25 was prepared by adjusting the pH of 0.25 N phosphoric acid to pH 2.25 with triethylamine. Twenty-microliter samples (approximately 1.48 μg protein) were injected directly onto the column, and a linear gradient of 16–34% B over 36 min was effective for separation of insulin from its metabolites.

Michaelis–Menten Kinetic Parameters from Initial Insulin Degradation Rates

The early degradation kinetics were examined to compare the rates at which trypsin and chymotrypsin attack the insulin molecule. Numerous initial experiments were conducted to determine the ratios of insulin to enzyme and incubation times necessary to produce a linear Lineweaver Burk plot and the expected asymptotic plot of velocity versus insulin concentration. Insulin solutions ranging from 1.7 × 10⁻⁵ to 1.45 × 10⁻³ M were incubated with either 1.0 × 10⁻⁷ M chymotrypsin or 8.4 × 10⁻⁷ M trypsin at 37°C since it was repeatedly observed that degradation by trypsin was less extensive than by chymotrypsin. Samples were taken at time 0 and at 1.5 min (for trypsin) or 2.5 min (for chymotrypsin). Under these experimental conditions the loss of insulin as a function of time was linear and the characteristic curve of velocity versus substrate concentration was evident. Samples were taken in triplicate, diluted with cold buffer to lower the pH to 2.5, refrigerated for 24 hr, and then assayed by HPLC in random order. For each concentration of insulin with each enzyme, linear regression of the starting and ending insulin concentrations produced the corresponding velocities of reaction.

Isolation and Identification of Early Insulin Metabolites of Chymotrypsin Degradation

When insulin was incubated with chymotrypsin and early samples were compared with those incubated for longer periods, five metabolite peaks which first appeared within 15–60 sec after the addition of enzyme were chosen for further analysis. Four experiments were run where insulin was incubated with chymotrypsin and numerous digest samples (100 μl each) were taken over the course of 5 to 9 hr, quenching the digestion by diluting it with cold Tris/TFA buffer. Observing the same peaks appearing/disappearing in the same ratios and over similar courses of time, larger amounts of insulin and chymotrypsin were incubated using the same molar ratio (344:1) and quenched at appropriate times so as to provide larger amounts of the initial metabolites for isolation. Then using the same C-8 analytical column as above, 2 ml of early digest samples (approximately 3 mg protein) was repeatedly injected onto the column and eluted with a TEAP 2.25/ACN gradient of 18–24% ACN over 48 min. The metabolite peak profiles produced from at least four separate insulin/chymotrypsin digests were the same as observed before at lower concentrations. The desired peaks were collected as they eluted. The separated metabolites were then individually chromatographed again using a second Rainin Microsorb C-8 column, 5 μm, 4.6 × 250 mm, but with gradients of water and ACN, each containing 0.1% (v/v)