Research Article

Degradation of Insulin by Trypsin and Alpha-Chymotrypsin

Robert J. Schilling[1] and Ashim K. Mitra[1,2]

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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 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 (1–4). 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.

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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 TPCK-treated trypsin from bovine pancreas, lyophilized type VII TLCK-treated 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 trifluoroacetic 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 μ*M* insulin solutions was prepared in a buffer composed of 100 m*M* Tris and 1 m*M* $CaCl_2$ 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 μ*M*. 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)

TFA. The TEAP 2.25/ACN gradient gave the best selectivity for the metabolites, especially those eluting near insulin, however, the water/ACN with TFA gradient removed small impurities and the metabolites were then in a volatile buffer system suitable for concentrating the samples for amino acid sequencing analysis.

Using a simplified chromatographic procedure, early samples of insulin chymotryptic digest were also chromatographed using TFA in water/ACN and appropriate fractions were collected. The metabolite fractions were dried at room temperature using a vacuum centrifuge to remove the HPLC mobile phase. Each metabolite was then dissolved in 25% formic acid and oxidized with an excess of performic acid as described by Sun and Smith (14) to oxidize the disulfide bridges in insulin and thus separate the A and B chains. The samples were again dried *in vacuo* and then analyzed by fast atom bombardment mass spectrometry. A computer program was used to match the mass of the observed peak with an amino acid sequence from insulin having that molecular weight.

RESULTS AND DISCUSSION

Insulin Degradation by Trypsin Versus Chymotrypsin

When equimolar ratios of insulin and enzyme were incubated at 37°C, pH 8.0, the loss of intact insulin was always substantially greater in experiments with chymotrypsin than with trypsin (Fig. 1). After 40 min of incubation with chymotrypsin, nearly 92% of insulin was lost, whereas only 7.5% of insulin was lost after 3 hr of incubation with trypsin. These experiments monitored the loss of intact insulin, therefore cleavage of any one bond would contribute to insulin loss. Trypsin and chymotrypsin are homologous serine proteases of similar size (near 24 and 25 kD, respectively). Only two bonds in insulin are susceptible to cleavage by trypsin (5,6). The end products of insulin digested with α-chymotrypsin have shown that a total of seven or eight bonds scattered throughout the A and B chains of insulin may be cleaved (1,3,4). To compare the activities of these two enzymes against insulin, assume that any of the bonds first cleaved in insulin may be randomly attacked [unlikely, since all bonds susceptible to chymotryptic cleavage are not exposed to the surface of the insulin molecule (17)]. If the enzymes were equally active against insulin and cleaved all bonds at the same rate, then at a given time for each enzyme

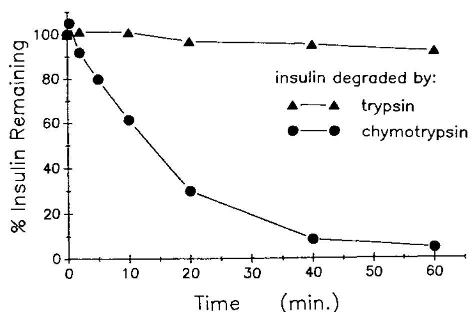


Fig. 1. Percentage insulin concentration remaining versus time when incubated with trypsin or chymotrypsin at 37°C, pH 8; 17.24 μM insulin was incubated with 0.1 μ*M* enzyme.

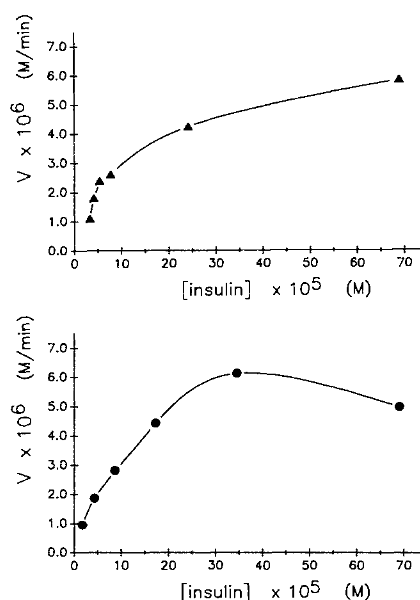


Fig. 2. Rates of initial insulin degradation versus insulin concentration with 8.4×10^{-7} *M* trypsin (top) and 1.0×10^{-7} *M* chymotrypsin (bottom). Triplicate measurements of concentrations (having standard deviations of approximately 5%) at times $t = 0$ and $t = 1.5$ or 2.5 min (for trypsin or chymotrypsin, respectively) were used to determine the velocities of reaction.

the loss of insulin divided by the number of bonds susceptible to cleavage by the given enzyme might be expected to be equal. In 40 min, chymotrypsin produced a 92% loss of insulin (with 8 susceptible bonds; 92/8 = 11.5% loss per susceptible bond in 40 min), whereas trypsin degraded only 5% of the insulin (with 2 susceptible bonds; 5/2 = 2.5% loss per susceptible bond in 40 minutes). Therefore, under these experimental conditions chymotrypsin was more active against insulin than trypsin and it is not due merely to more sites being susceptible to chymotryptic cleavage, especially considering the fact that all bonds susceptible to chymotryptic cleavage are not exposed in the native insulin molecule and thus not equally accessible for initial cleavage.

To examine further the difference in intrinsic activities of these two enzymes, their rates of bond cleavage using

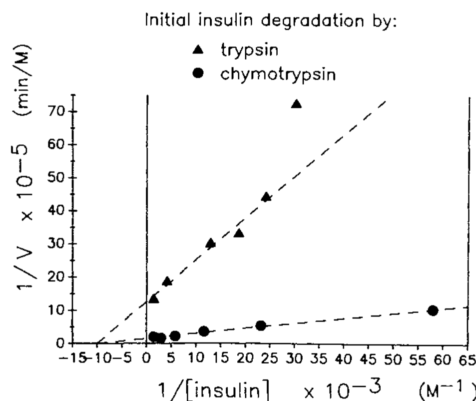


Fig. 3. Lineweaver Burk plot of initial insulin degradation by trypsin and chymotrypsin, normalized for equal enzyme concentrations.

Table I. Michaelis–Menten Kinetic Parameters Determined for Initial Insulin Degradation by TLCK-Treated α-Chymotrypsin and TPCK-Treated Trypsin[a]

Enzyme	Apparent K_m (mM)	Apparent V_{max} (M/min)
α-Chymotrypsin	0.100	6.48×10^{-6}
Trypsin	0.099	0.75×10^{-6}

[a] Range of insulin concentrations employed was 1.7×10^{-5}–1.45×10^{-3} M. Data normalized for enzyme concentrations of 1.0×10^{-7} M.

model compounds were compared. Using the experimental protocol developed at Sigma Chemical Company, the cleavage of BAEE by trypsin produced the expected change in UV absorbance as predicted by the activity as specified on the label by the manufacturer. To convert the BAEE units of activity into a rate of bond cleavage, the absorbances of known mixtures of BAEE and benzoylarginine were measured to prepare a standard curve. The 12,000 BAEE U/mg specified for trypsin was found to correspond to a rate of hydrolysis of 0.314 μmol/min. The activity of the chymotrypsin, on the other hand, was specified as the hydrolysis of benzoyltyrosine-ethyl ester at a considerably faster rate of 57 μmol/min. This indicates a greater intrinsic activity for chymotrypsin over trypsin, and this concurs with the greater degradation of insulin observed with chymotrypsin.

Michaelis–Menten Kinetic Parameters of Early Insulin Degradation

It was hypothesized that the prevention of initial bond cleavage in insulin may also prevent subsequent bond hydrolysis, thus the kinetic parameters of early insulin degradation were examined in more detail. Insulin solutions used ranged in concentrations from 1.7×10^{-5} to 1.45×10^{-3} M. It was found that 1.0×10^{-7} M chymotrypsin produced plots that would be expected of catalytic processes following Michaelis–Menten kinetics and since the rate of cleavage by trypsin was much lower, 8.4×10^{-7} M trypsin was employed (Fig. 2). Lineweaver Burk plots of insulin degradation by chymotrypsin were plotted directly, while the rates of insulin degradation by trypsin were divided by 8.4 to nor-

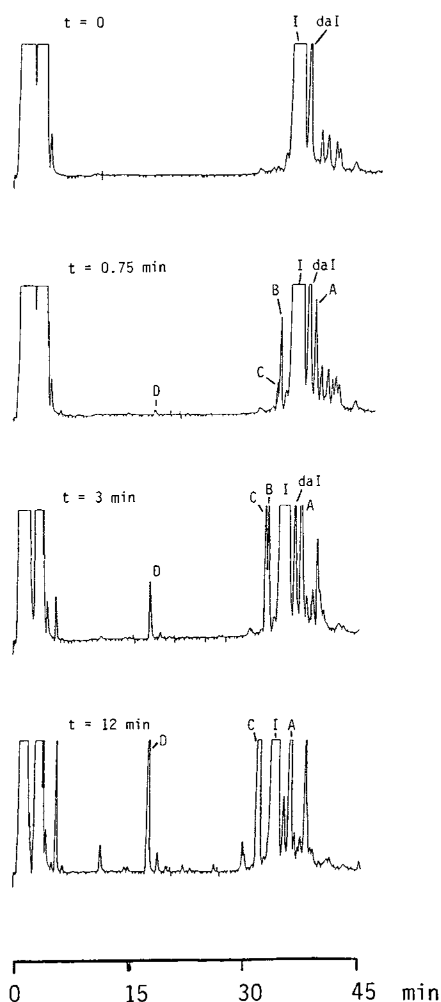


Fig. 4. Example of HPLC separation used for the collection of early metabolites from insulin/chymotrypsin digests. Insulin was incubated with chymotrypsin (344:1 molar ratio) and 2 ml of digest samples was injected onto a C-8 column and eluted with a gradient of TEAP 2.25/ACN as in text. A, metabolite A; B, metabolite B; C, metabolite C; D, metabolite D; I, insulin; daI, desamido-insulin.

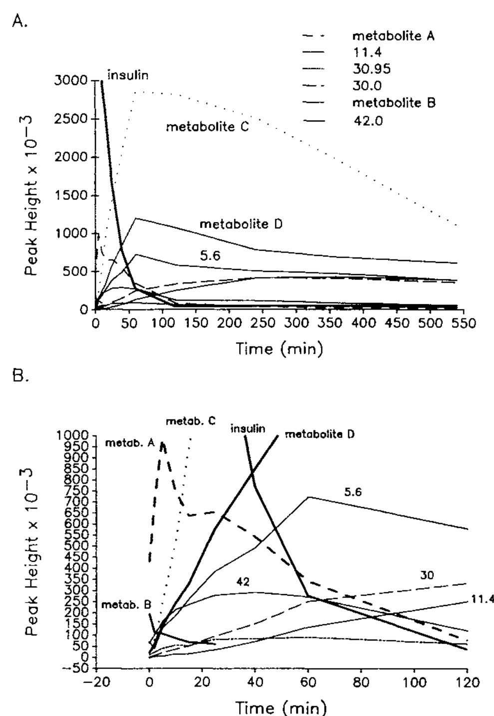


Fig. 5. Example of profile of HPLC peak heights of insulin and metabolites versus time when insulin was incubated with chymotrypsin (344:1 molar ratio). Samples were taken at 0, 2, 5, 10, 15, 25, 40, 60, 120, 360, and 540 min. Twenty microliters of digest sample was injected onto a C-8 column and eluted with a gradient of TEAP 2.25/ACN as in text. B is a section of A enlarged to show greater detail of early events. Smaller or later-appearing metabolites which were not identified are denoted with numbers corresponding to their retention time.

Table II. Amino Acid Sequencing Results for Insulin Metabolites Isolated First Using an HPLC Gradient of TEAP/ACN and Additionally Purified with an HPLC Gradient of TFA in Water/ACN

Cycle No.	Residue(s) identified[a]	Concentration (pmol)
	Metabolite A	
1	Gly/Phe/Asp	444/477/140
2	Ile/Val	176/223
3	Val/Asn	249/418
4	Glu/Gln	376/380
5	Gln/His	385/NQ[b]
6	Leu	331
7	Tyr/Cys	35/NQ
8	Thr/Gly	100/226
9	Ser	120
10	Ile/His	61/NQ
11	?/Leu	NQ/187
12	Ser/Val	31/108
13	Leu/Glu	99/119
14	Tyr/Ala	39/166
15	Gln/Leu	73/NQ
16	Leu/Tyr	NQ/NQ
17	Glu/Leu	84/124
18	Asn/Val	88/96
19	Tyr/Cys	77/NQ
20	Cys/Gly	NQ/91
21	Asn/Glu	40/75
22	Arg	39
23	Gly	67
24	Phe	50
25	Phe	54
26	Tyr	7
	Metabolite B	
1	Gly/Phe	424/517
2	Ile/Val	202/237
3	Val/Asn	262/436
4	Glu/Gln	428/402
5	Gln/His	417/351
6	Leu	368
7	Tyr/Ser?	52/3
8	Thr/Gly	245/NQ
9	Ser	198
10	Ile/His	74/226
11	?/Leu	NQ/207
12	Cys?/Val	NQ/124
13	Leu/Glu	104/155
14	Tyr/Ala	58/194
15	Gln/Leu	77/194
16	Leu/Tyr	130/129
17	Glu/Leu	54/227
18	Asn/Val/Cys?	64/95/NQ
19	Tyr	50
20	Gly	105
21	Glu	99
22	Arg	59
23	Gly	84
24	Phe	60
25	Phe	80
26	Tyr	12

Table II. Continued

Cycle No.	Residue(s) identified[a]	Concentration (pmol)
	Metabolite C	
1	Gly/Phe/Asp	102/110/837
2	Ile/Val/Arg?	43/76/45
3	Val/Asn	82/88
4	Glu/Gln	77/88
5	Gln/His	105/19
6	Leu	77
7	Tyr/His	9/9
8	Thr/Gly	17/37
9	Ser	34
10	Ile/His	22/NQ
11	Leu	46
12	Ser/Val	10/34
13	Leu/Glu	22/40
14	Tyr/Ala	9/31
15	Leu	35
16	Tyr	17
	Metabolite D	
1	Gln/Leu/Asp	230/629/79
2	Cys?/Val	NQ/316
3	Glu	146
4	Asn/Gly	187/413
5	Tyr/Glu	353/76
6	Cys/Arg	NQ/NQ
7	Asn/Gly	82/205
8	Phe	124
9	Phe	112

[a] The amino acid identified from the insulin A chain is listed first, that from the B chain is listed second, and any additional (interference) peak is listed third.
[b] Not quantified.

malize for the higher enzyme concentration employed (Fig. 3). Table I summarizes the apparent K_m and V_{max} calculated from this Lineweaver Burk plot. The data suggest that the apparent K_m's for the two enzymes were nearly the same (0.1 m*M*), while the apparent V_{max} for the initial degradation by chymotrypsin was 8.64 times greater than for trypsin.

Identification of Early Metabolites from Chymotrypsin Degradation

Trypsin, as stated earlier, cleaves at two sites in insulin, both of which are relatively well exposed to the surface (17). While cleavage at B29-Lys does not inactivate insulin, the second cleavage site (after B22-Arg) inactivates insulin (5,6) and protection would be desired. Knowing this, and having seen that chymotrypsin is much more active against insulin, it was of interest to determine whether any of the bonds in insulin are especially susceptible to initial cleavage by chymotrypsin. Using an HPLC gradient of TEAP/ACN, two new peaks were observed after only 20 sec of incubation of insulin and chymotrypsin in repeated studies. Within 1 min, a total of five new peaks was apparent on the chromatogram, four of which were prominent (Fig. 4). Additional peaks also appeared over the course of time (Fig. 5). It should be noted

that these profiles represent only relative amounts of metabolites (expressed as peak heights), since the metabolites themselves could not be quantitated with a standard curve, and their absorbance coefficients cannot be assumed to be equal. The appearance and disappearance of some of the peaks with time may be indicative of some sequential as well as parallel events of hydrolysis.

Collection of these initial metabolite fractions and analysis by amino acid sequencing (Table II) revealed the following cleavage sites, with a proposed pathway shown in Scheme I. Sequencing the first two metabolites to appear on HPLC showed that cleavage occurred after B26-Tyr (metabolite A) and also after both B26-Tyr and A19-Tyr (metabolite B). The formation of metabolite A and metabolite B may be sequential, although both tyrosine residues are exposed on the surface of the insulin molecule (17) and thus both may be easily attacked, perhaps simultaneously. Additionally, metabolite A is apparently degraded in a parallel reaction to form metabolites C and D. Three bonds were cleaved in this process, which may occur simultaneously as no intermediates were observed. Cleavage occurred, then, after A14-Tyr and B16-Tyr to form metabolite C and metabolite D, the latter of which also had cleavage after B25-Phe.

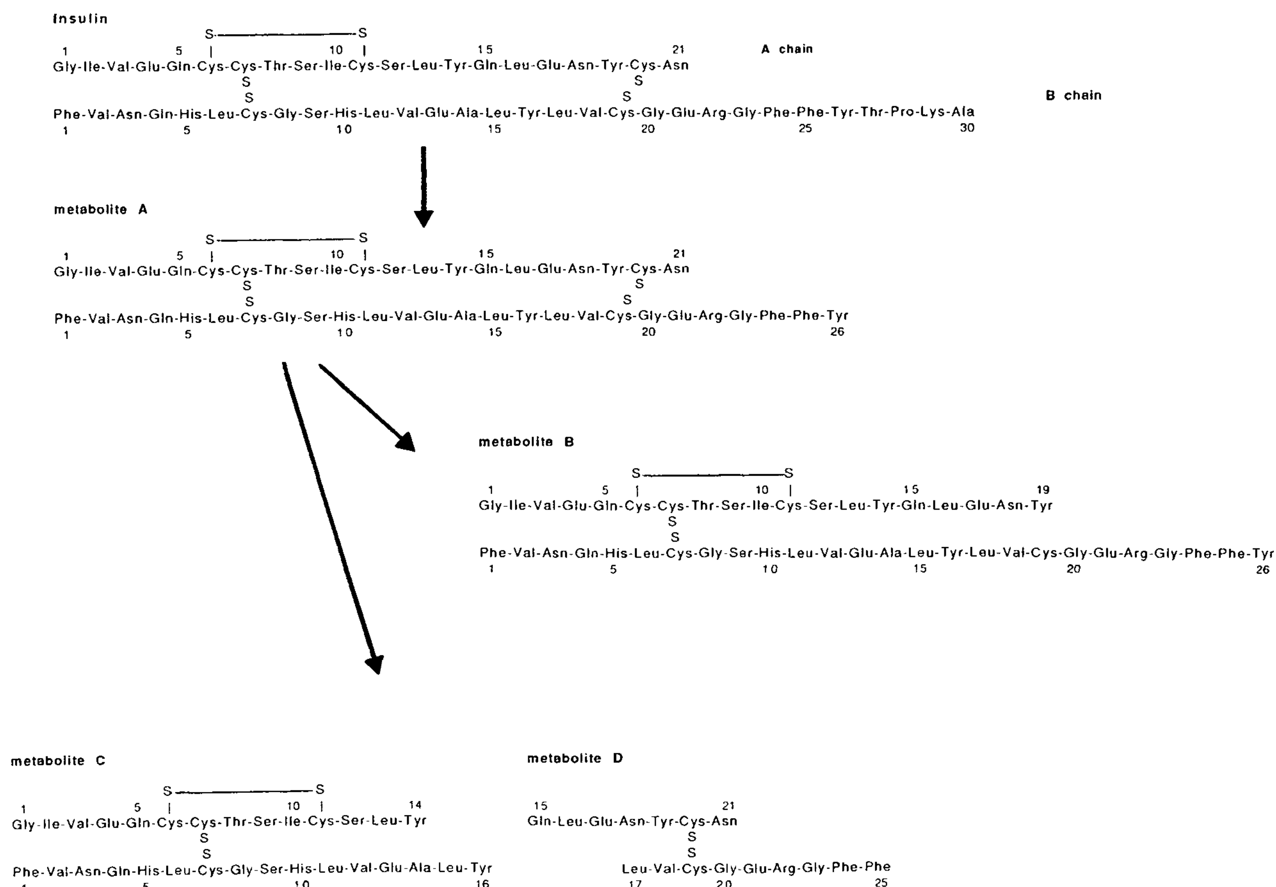
Additional evidence for the structures of these metabolites was obtained from FAB mass spectra (Table III). After chromatographic separation, the isolated metabolite fractions were oxidized with performic acid and this resulted in a number of nonspecific cleavages. However, peaks were observed with m/e's corresponding to the A and B chains of metabolite A and metabolite C and to the B chain of metabolite D. The difficulty of detecting the insulin A chain in FABMS has been noted previously (18). Also, since metabolite B was observed to decrease in concentration very rapidly in the chymotryptic digestion of insulin, it is very likely that this metabolite was not present in the samples in sufficient quantity to be detected by FABMS.

The original proposal of this research followed the hypothesis that the slow step of insulin degradation by chymo-

Table III. Mass of the Molecular Ions in the FAB Mass Spectra of Insulin Metabolites Which Were First Separated by HPLC (Using a TFA Gradient) and Then Oxidized with Performic Acid

Mass[a]	Segment	Corresponding segment from amino acid sequencing
2572	A_1–A_{21}	Metabolite A
3098	B_1–B_{26}	
1662	A_1–A_{14}	Metabolite C
1877	B_1–B_{16}	
—[b]	A_{15}–A_{21}	Metabolite D
1075	B_{17}–B_{25}	

[a] Mass (m/e) reflects oxidation of cysteine and cystine to cysteic acid.
[b] Not observed.



Scheme I. Initial degradation of insulin by α-chymotrypsin.

trypsin was the interaction of insulin with the enzyme for the first bond cleavage. Preventing this first step could protect insulin from degradation. The results obtained here, however, show that in a very short time five bonds are hydrolyzed. Studies of the end products of chymotryptic digestion of insulin have shown that only two or three additional bonds (depending on experimental conditions of these investigators) remain to be cleaved: those after B1-Phe, B15-Leu, and A11-Cys (1,3,4). Thus the simple idea of protecting the "initial" site of cleavage to render the insulin resistant to degradation becomes rather complex. Indeed, the five amino acids at which chymotrypsin initially cleaved in these experiments are all exposed to the surface of the molecule (17) and therefore relatively accessible for attack. Of the other sites susceptible to cleavage in long-term incubations, the B15-Leu and A11-Cys residues are localized in the hydrophobic core of the insulin molecule (17) and, as a result, were not cleavage sites in initial degradation. Protection of internal sites susceptible to cleavage might then be achieved by preventing hydrolysis at sites of initial cleavage, however, this would be more feasible in a protein molecule having fewer sites exposed for initial cleavage than insulin, since fewer alterations would be less detrimental to the biological activity of the molecule.

If only one or two bonds would have to be protected by such proposed alterations, it might be possible to design an insulin analogue which retains good biological activity and is resistant to chymotrypsin. With the necessity to alter up to five bonds in insulin, however, it would be less feasible to achieve both adequate protection and bioactivity. Four of the five residues of concern (B16-Tyr, B25-Phe, B26-Tyr, and A19-Tyr) have long been implicated as among those important or essential for receptor binding (19). These four residues are invariant among many different species (except B25-Phe, which is invariant as an aromatic amino acid), which further implies the important role that they play in maintaining the biological activity of insulin (17). A number of modifications to sites on insulin such as these have been examined. For our purposes, these modifications could prevent hydrolysis at the respective sites, however, some of the changes made to the insulin molecule resulted in a drastic reduction of biological activity (20–24). Thus, each susceptible cleavage site will have to be examined individually to determine whether it is possible to alter it subtly without grossly changing its character required for insulin activity.

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