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Synthesis and Characterization of Insulin-Transferrin Conjugates

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Abstract

Receptor- mediated endocytosis can be exploited for improving the transcellular delivery of therapeutic proteins. Insulin conjugated to transferrin by forming disulfide bonds has been shown to improve insulin oral bioavailability in diabetic rats. We are developing a combination strategy involving complexation hydrogels as delivery vehicles for insulin- transferrin conjugates. The complexation hydrogels developed in our laboratory have been shown to be promising carriers for oral delivery of proteins and peptides. Integrating the strategies based on the complexation hydrogels and insulin-transferrin conjugates may prove to be a novel approach for oral delivery of insulin, and other therapeutic proteins. In this work, electrospray ionization mass spectrometry (ESI-MS) was used to study the modification of insulin during its reaction with transferrin. The stability of the conjugated insulin to ezymatic degradation was also studied. ESI-MS studies confirmed the site-specific modifications of insulin. The transferrin-conjugation of insulin was also shown to increase the stability of insulin to enzymatic degradation.

INTRODUCTION

Complexation hydrogels developed in our laboratory have emerged as one of the most promising methods for orally delivering therapeutic proteins (1–3). These hydrogels are hydrogen bonding, copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol), henceforth designated as P(MAA-g-EG) hydrogels. Their pH-responsive behavior resulting from the hydrogen bonding complexation/decomplexation characteristics makes them ideal carriers for oral delivery of proteins, such as insulin. Insulin entrapped inside the polymer network is protected in the harsh gastric environment before it is released in the upper small intestine, from where it is absorbed into the systemic circulation. Further, the polymer network can inhibit the activity of Ca^{2+} dependent proteolytic enzymes. Both in vitro and in vivo studies with insulin loaded polymer carriers have demonstrated the efficacy of these hydrogels as vehicles for oral administration of insulin (1,4,5).

One of the other effective strategies for enhancing bioavailability of proteins exploits the receptor- mediated endocytotic pathway used by the cells for selective and efficient uptake of specific macromolecules required for various cell processes. By coupling proteins and peptides to ligands that can recognize specific receptors on the epithelial cells, transcellular delivery of macromolecular biopharmaceuticals may be achieved. Since only those molecules that are conjugated to the ligands are transcytosed, this process eliminates potential side effects associated with the unspecific transport via the paracellular pathway.

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Transferrin is a widely investigated protein for enhancing the cellular uptake of therapeutic agents. It is a naturally occurring protein involved in iron transport. Absorptive uptake of the iron-bound transferrin has been used to enhance the transcytosis of therapeutic agents and drug carriers across polarized cells such as the epithelial cells (12,13) and the endothelial cells (6–9). Transferrin receptor (TfR)-mediated cellular uptake has also been explo red for targeted delivery of anticancer drugs, proteins, and therapeutic genes into primary proliferating malignant (10,11).

In one promising application of TfR- mediated drug delivery, Shen and co-workers (12,13) recently demonstrated improved efficacy of orally administered insulin by conjugating insulin with transferrin through disulfide linkages (12). Shah and Shen (13) showed that insulin conjugated to transferrin via disulfide linkages can be transported across enterocytes- like Caco-2 cells. Further, Xia et al. (12) showed that orally administered insulin-transferrin conjugate exhibited a slow but prolonged hypoglycemic effect compared to that of the native human insulin in streptozotocin- induced diabetic rats. One important consideration relating to the use of TfR-conjugated proteins for improved oral bioavailability is that the TfR is primarily present in the basolateral membrane of the epithelial cells (14). This may limit the desired apical to basolateral transcytosis of the conjugated molecule. Despite this limitation, successful studies conducted by Shen and coworkers have demonstrated that insulin and granulocyte-colony stimulating factor (GCSF) conjugated to transferrin were transported across Caco-2 cell monolayers (13,15). Transcytosis experiments with radio- labeled insulin demonstrated a 15-fold (apical-to-basolateral) and five- fold (basolateral-to-apical) increases in insulin transport compared with unmodified insulin across Caco-2 cells (13). This transport was TfR- mediated because it was efficiently inhibited by unlabeled Tf (13). This indicates that the distribution of the TfRs on the apical side may be sufficient to cause significant enhancement in the transport of insulin across the epithelial cell barrier.

In our laboratory, we are developing a delivery system consisting of the complexation hydrogels acting as delivery vehicles for insulin-transferrin conjugates. The use of P(MAA-g-EG)-based microparticles as delivery vehicles for the insulin-transferrin conjugates may constitute a superior transmucosal delivery system for insulin. The system may improve the efficacy of oral insulin administration since: (i) insulin in the conjugated form may be further protected from enzymatic degradation due to steric hindrance created by the conjugated transferrin; (ii) owing to the potential of complexation hydrogels to act as mucoadhesive carriers, most of the conjugate will be released within the localized microenvironment of the small intestine's filamentous brush border creating a high local concentration of the conjugate; (iii) the conjugate will have larger residence time in the small intestine; and (iv) the insulin-transferrin conjugate can cross the intestinal barrier by TfR- mediated transcellular pathway, which may further increase the efficiency of insulin absorption.

In this work we discuss the synthesis of insulin- transferrin conjugates, analysis of the intermediate products, and characterization of the reaction products. Detailed analysis of the insulin modification reaction and determination of stability to enzymatic degradation of the conjugated insulin are important steps in developing the proposed combination strategy.

EXPERIMENTAL SECTION

Materials

The heterobifunctional crosslinker, succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and dithiothreitol (DTT) were purchased from Pierce (Rockford, IL). Dimethylmaleic anhydride (DMMA) was purchased from Sigma-Aldrich (Chemie, Steinheim, Germany). Bovine insulin, trypsin and 4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′-dione (fluorescamine) were purchased from Sigma (St. Louis, MO). Float-a-Lyzer® dialysis tubings were purchased from

Spectrum Laboratories (Rancho Dominguez, CA). Slide-A-Lyzer® Mini Dialysis units, D-Salt Dextran Desalting Columns and immobilized trypsin, TPCK, were purchased from Pierce Biotechnology, Inc. (Rockford, IL). N-ethylmaleimide (NEM) was obtained from MP Biomedicals (Aurora, OH). C18 ZipTips were purchased from Millipore (Billerica, MA).

Synthesis of Insulin-Transferrin Conjugates

The protein conjugation method described here has been developed by Carlsson et al. (16) and was modified for preparation of insulin- transferrin conjugates by Shah and Shen (13). Insulin (Ins) was conjugated to transferrin (Tf) through disulfide linkages using SPDP, according to the method described by Shah and Shen (13) with some modifications as described below.

Insulin Modification by DMMA (Step I)

Insulin was reacted with (DMMA) to block the primary amine groups at the n-terminals of insulin chains which can potentially react with SPDP (Figure 1A). A sample of 25 mg of bovine insulin was added to a solution containing 1.5 mL of 1N HCl and 3 mL phosphate buffered saline (PBS) solution (pH 6.9). After completely dissolving insulin, the pH of the solution was adjusted to 6.9 by addition of 1.5 mL of 1N NaOH solution. Dissolved insulin was then reacted with 3.5 mg of DMMA under constant stirring for 0.5 h. The pH of the solution was maintained at 6.8 to 6.9 by addition of 1M Na_2CO_3 solution. Reaction with DMMA was repeated two more times so that a total of 10.5 mg of DMMA was added to insulin at the controlled pH of 6.8–6.9. A micro-pH meter (IQ Scientific Instruments, San Diego, CA) was used to monitor the pH of the solution during the reaction. The reaction mixture was transferred to Float-a-Lyzer® dialysis tubings (MWCO 3500) and dialyzed against PBS buffer (pH 6.9) for 24 hours to remove unreacted DMMA.

Insulin Modification with SPDP (Step II)

Following dialysis, the DMMA- modified insulin was reacted with SPDP to introduce disulfide bonds at primary amine group of lysine at the B29 position of insulin (Figure 1B). The pH of modified insulin solution was raised to 9.0 by addition of 1M Na_2CO_3 and 6.0 mg of SPDP dissolved in 150 μL dimethyl formamide (DMF) was added to this solution. The reaction was constantly stirred at 4 °C for 2 hours. The pH of the reaction was maintained at 8.8–9.0 during the course of the reaction.

The modified insulin was then dialyzed against PBS buffer (pH 8.0) for 24 hours to remove unreacted SPDP reagent. To measure the average number of moles of 2-pyridyl disulfide groups attached per mole of insulin (PDP: Ins ratio), 700 μL of Ins-PDP solution was reduced by 25mM DTT. PDP- modified protein on reduction with DTT releases of 2-pyridinethione chromophore which can be quantitated by measuring the absorbance at 343 nm (A_{343}). A molar extinction coefficient of 8.08 × 103 $M^{-1}cm^{-1}$ at 343 nm (e_{343},2-TP) and 5.10 × 103 $M^{-1}cm^{-1}$ at 280 nm (e_{280},2-TP) was used for the calculations (16,17). Protein concentration was determined by measuring the absorbance at 280 nm (A_{280}). The molar extinction coefficient of 5.8 × 103 $M^{-1}cm^{-1}$at 280 nm was used for insulin. A sample of 40 μL DTT reduced modified insulin solution was diluted to 700 μL and the absorbance was measured in triplicates at 343 nm and 280 nm with a microplate reader (Synergy HT, Bio-Tech Instruments, Winooski, VT). The PDP: protein ratio was determined by the following formula.

$$\mathrm{PDP/protein} = \frac{A_{343} \times e_{280,\,\mathrm{protein}}}{(A_{280} \times e_{343,2-\mathrm{TP}} - A_{343} \times e_{280,2-\mathrm{TP}})} \quad (1)$$

Transferrin Modification with SPDP (step III)

Transferrin was dissolved in 4 mL phosphate buffer (pH 7.0, 30mM) at a concentration of 20 mg/mL. Four- fold molar excess of SPDP dissolved in 100 μl DMF and added to the transferrin solution under constant stirring. The reaction mixture was stirred for 2 hours at 4 °C. The modified transferrin was then dialyzed (MWCO 50,000) against phosphate buffer (pH 8.0, 30mM, 3mM EDTA) for 24 hours to remove unreacted SPDP reagent. Following dialysis, entire transferrin solution was reduced by 25mM DTT for 1 h to generate thiol-containing transferring, Tf-SH (Figure 1C). The PDP: Tf modification ratio was determined by using molar extinction coefficient of $93.0 \times 10^3\ M^{-1}cm^{-1}$ at 280 nm (e_{280},Tf) as described above.

Conjugation of Modified Proteins (Step IV)

The reaction solution from step III containing Tf-SH was immediately purified by elution on D-Salt Dextran Desalting Columns (MWCO 5000). Four columns were connected in series to achieve the desired purification. Phosphate buffer (pH 8.0, 30 mM, 3 mM EDTA) was used to elute the protein from the columns. Fractions with A_{280} values greater than 0.2 were collected.

Purified Tf-SH was then reacted with Ins-PDP (Figure 1D) in a molar ratio of 1:2 of Tf-SH: Ins-PDP. To follow the progress of the reaction, 40 μL of the reaction samples were withdrawn at predetermined time intervals and diluted to 700 μL with phosphate buffer. Reaction of each mole of insulin with Tf-SH is accompanied by the release of one mole of 2-pyridinethione. Hence, A_{343} values of the samples were measured with a microplate reader to determine the concentration of 2-pyridinethione. To determine the average number of moles of insulin attached to one mole transferrin (Ins: Tf ratio), concentration of the 2-pyridinethione was divided by transferrin concentration in the samples. When approximately 2 moles of 2-pyridinethione were released per mole of transferrin, the reaction was stopped by addition of 4 mg NEM to prevent further crosslinking of transferrin molecules via oxidation of unreacted thiols (13). Following the reaction, the conjugate was purified by dialysis (MWCO 25,000) against phosphate buffer (pH 8.0, 30mM, 3mM EDTA) until no free insulin was detected in the solution by HPLC. Total protein concentration of the final purified conjugate solution was determined by Quick Start™ Bradford assay (Bio-Rad Laboratories, Hercules, CA) using BSA or unmodified transferrin as a reference standard (18).

Analysis of the Insulin Modification Reaction

Modifications of the primary amines of insulin due to DMMA reaction (step I) and SPDP modification (step II) was studied by quantitatively measuring the amount of free primary amine groups of the protein using fluorescamine assay and by identifying the sites of insulin modification by mass spectrometry. This was important since improper modifications of the protein may lead to decrease n its bioactivity (19).

Fluorescamine Assay for Measurement of Free Amine Groups of Insulin

A primary amine assay utilizing fluorescamine was performed to estimate insulin primary amine modification by DMMA and by SPDP. 1.08 mM fluorescamine stock solution was prepared by dissolving 0.9 mg fluorescamine in 3 mL acetone. Insulin standard solutions were prepared containing 0.05, 0.1, 0.25, 0.5, 1, and 2 mg/mL native, unmodified insulin in PBS buffer. 50 μL of fluorescamine stock solution was added to 150 μL of insulin standard solutions in 96 well plates and the fluorescence measurements were performed a microplate reader (20). The excitation wavelength for measurements was 390 nm and the emission wavele ngth was 470 nm. A standard curve of emission at 470 nm (Em470) vs. insulin concentration (mg/mL) was prepared (Figure 2).

Samples were taken from the modification reaction before modification with DMMA (after step I), after purifying the DMMA- modified insulin and after purifying the SPDP-modified insulin intermediate (after step II). The concentrations of the samples were adjusted to account for the dilution caused by dialysis of the modified intermediate products, so that the final concentration in all the samples was 0.75 mg/mL. A plot of Em470 vs. concentration (mg/mL) was prepared for all the three samples. The slopes of standard curve and plots for three samples were calculated and plotted against the expected number of moles of free amines present per mole of insulin samples (Figure 3).

Mass Spectroscopy Analysis of the Insulin Modification Reaction

Sample aliquots were taken before and after the DMMA and SPDP modification steps. Dialysis of each aliquot was performed by Slide-A-Lyzer® Mini Dialysis units (3500 MWCO) against 0.5 L deionized water with Triethylamine (TEA) at pH 7.5 for 30 minutes. The dialysis was repeated before dilution for ESI-MS analysis. SPDP modified insulin was first dialyzed than digested with immobilized trypsin beads treated with 0.1M ammonium acetate buffer (pH 8.5) for 18 hours at 37°C. The trypsin digest samples were desalted and concentrated with C_{18} ZipTips®. ESI-MS and MS^n experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose, Ca). All the samples were diluted to 10 μM and run at 3μL/min for ESI-MS analysis in 99/1/1 methanol/water/TEA ($MeOH/H_2O$/TEA) by volume for negative mode or 99/1/1 methanol/water/acetic acid ($MeOH/H_2O$/HoAc) by volume for the positive mode unless otherwise noted. All the chemicals were purchased from Sigma (St. Louis, Mo) without any further purification unless otherwise noted.

HPLC Analysis of the Insulin-Transferrin Conjugates

A reversed phase-HPLC method was established for analysis of the conjugates to be used in quantification of the conjugates in later studies. The same method was used for measurement of insulin and transferrin concentrations. The HPLC system was a Waters 2695 Alliance Separation Module (Waters, Milford, MA) equipped with Waters 2487 Dual ? Absorbance Detector (Waters, Milford, MA). All the data were collected utilizing the Millenium Software Empower® Application. A Symmetry300™ C4 column (particle size 5 μm, 3.9mm i.d. ×150mm length) (Milford, MA) was used for the separations. The solvents used for the analysis were: solvent A (water, 0.1% trifluoroacetic acid (TFA)) and solvent B (HPLC-grade acetonitrile, 0.08% TFA). The mobile phase for the analysis consisted of a linear gradient of 70% of solvent A to 40% of solvent A in 6 min. The gradient was controlled by the Empower software. The column temperature was set at 40 °C. The flow rate of the mobile phase was set at 0.6 mL/min and the peaks were detected at 215 nm.

Analysis of Stability of the Insulin-Transferrin Conjugates to Enzymatic Degradation

The degradation profile of the conjugates was studied in comparison with insulin and transferrin in the presence of trypsin, a major proteolytic enzyme present in the intestine. The enzyme solution was prepared by dissolving trypsin in 50 mM phosphate buffer solution with pH of 7.4 and ionic strength of 154 mM. Insulin and transferrin stock solutions were prepared in the reaction buffer. Conjugate solution was diluted with the reaction buffer to achieve desired concentration. All the solutions were pre-warmed at 37 °C for 15 minutes. The protein and the trypsin concentrations in the stock solutions were adjusted so that after adding trypsin solution to the insulin, transferrin and conjugate stock solutions, the initial protein concentration in the reaction mixture was 1 mg/mL and the trypsin concentration was 3.2 mg/mL. At time t = 0, trypsin stock solution was added to insulin, transferrin and the conjugate solutions at 37 °C under continuous mixing. 50 μL samples were withdrawn at predetermined time intervals and the enzyme activity in the samples was stopped by addition of 50 μL ice-cold acetic acid solution (50% (v/v) acetic acid in DI water). The samples were then analyzed by HPLC as

described previously. Degradation rate constants were determined from the slope of semi- log plot of the percentage of initial protein remaining vs. time.

RESULTS AND DISCUSSION

Synthesis of Insulin-Transferrin Conjugates

Insulin was conjugated to transferrin using SPDP, a heterobifunctional, cleavable cross- linker that reacts with primary amines and sulfhydryl groups (21) (Figure 1). Upon SPDP reaction with the accessible primary amines of a protein, a mixed disulfide is formed which has free 2-pyridyldithio groups, that can react with free sulfhydryl groups from other proteins. This results in formation of conjugates of different proteins (heteroconjugates), without unwanted cross-reaction products such as the homoconjugates of individual proteins. This crosslinker is especially applicable to this research since the disulfide linkages are inherently unstable in the plasma (22). Hence disulfide linkages linking the two proteins may be cleaved once the conjugate reaches systemic circulation (18,23,24). Xia et al. (12) showed that free insulin was released within 5 minutes of incubation of the insulin-transferrin conjugates with rat liver slices. Free insulin was also detected in the rat serum 4 hours after oral administration of the insulin-transferrin conjugates (12).

Step I of the conjugation involves modification of the n-terminal primary amines of insulin by reaction with DMMA. Insulin has three primary amine moieties: at A1-Glycine (A1Gly), B1-Phenylalanine (B1Phe) and at B29-Lysine (B29-Lys) (Figure 7). All the primary amines can potentially react with SPDP reagent at a pH where most of the amine groups are deprotonated so that the $–NH_2$ group can act as a nucleophile and react with the electron-deficient carbon atom of the succinimidyl group of SPDP. However, Lindsay and Shall (25) reported that acetylation of insulin at the A1Gly position resulted in decrease in the biological activity of insulin. Hence insulin modifications involving primary amines are usually directed to either B1Phe or B29Lys positions. In this work, SPDP modification was carrier out at the B29 lysine group of insulin. Hence it was necessary to block the n-terminal primary amines before reacting insulin with SPDP. This was done by reacting insulin with DMMA at a controlled pH of 6.8–6.9 (12). At this pH, most of the terminal amine groups were in their deprotonated state, and were able to react with DMMA through nucleophilic attack. However, since the pH was about two units below the pKa of the lysine residue, most of the lysine residues remained unreacted. The DMMA modification of insulin is acid- labile and the amino acid residues can be regenerated by making the solution slightly acidic.

SPDP modification of the purified intermediate from step I was carried out at the pH of 9.0 to generate the Ins-PDP intermediate. The modification was measured by UV spectroscopy after reducing a small aliquot of purified Ins-PDP by 25 mM DTT. The modification ratio (PDP: Ins) calculated from Equation 1 was approximately 1: 1. Modification ratio for transferrin (PDP: Tf) was approximately 4: 1.

After reducing the Tf-PDP intermediate to generate thiol containing transferrin (Tf-SH), the intermediate was purified by size exclusion chromatography. Two moles of Ins-PDP were then added per mole of Tf-SH. When added Ins-PDP was almost entirely reacted releasing two moles of 2-pyridinethio per mole of transferrin, the reaction was stopped by addition of NEM to prevent crosslinking of the Ins-Tf conjugates due to oxidation of excess –SH on insulin-conjugated transferrin. The conjugation ratio of 2:1 Ins:Tf was chosen since excessive modification of transferrin by insulin attachment will result in a bulky complex which may adversely affect the diffusion characteristics of the conjugate in the polymer network reducing loading and release efficiencies of the conjugate.

Little or no insulin was detected in the reaction mixture by HPLC at the end of the reaction. The conjugate was extensively dialyzed to remove any unreacted residual insulin. The concentration of purified conjugate was determined using Bradford assay (18). Because many proteins have nearly identical response curve in this assay, the methods can be applied widely using a single set of standards (18). BSA and transferrin were used as standards to calculate the concentration of conjugate in the purified conjugate solution.

Analysis of the Insulin Modification Reaction

Cellular studies performed by Shah and Shen (13) showed that conjugate molecules containing up to three insulin molecules per molecule of transferrin were able to be transported across the Caco-2 cell monolayers by a transcellular mechanism. This means that the SPDP modification of the surface exposed lysine residues of transferrin did not decrease its ability to bind the transferrin receptors on the cell surfaces nor inhibit transport across the cell monolayers by receptor-mediated transcytosis. However, improper modification of insulin by DMMA or SPDP may result in inhibition of its physiological activity. Hence sites of modification of insulin were verified here by fluorescamine assay and mass spectrometry analysis of the insulin modified by step I (DMMA modification) and step II (SPDP modification).

Fluorescamine Assay for Quantification of the Primary Amine Groups

Fluorescamine, a non- fluorescent compound, reacts rapidly with primary amines in proteins, such as the terminal amino groups and the e-amino groups of lysine residues, to form fluorophors which are excited at 390 nm and fluoresce at 475 nm (26). The reaction is completed in 100–500 ms and excess reagent is quickly hydrolyzed to nonfluorescent products. The fluorescent product is stable for several hours.

Figure 2 shows the plot of Em470 vs. concentration for insulin standards, unmodified insulin (before step I), DMMA- modified insulin (after step I), and SPDP-modified insulin (after step II). As can be seen from the figure, the calibration plots for standard curve and unmodified insulin overlap and have comparable slopes. The slopes of the curves are greatly reduced after DMMA modification, indicating that the number of free amino groups per mole of insulin has decreased. Fluorescence values for corresponding concentrations of the SPDP- modified insulin are even lower indicating that the SPDP-modified derivative of insulin had fewer amino groups that are free to react with fluorescamine than the DMMA- modified insulin.

Ideally, PDP- insulin should not react with fluorescamine since a successfully modified insulin molecule should not have any free amino groups. But in actual reaction, some unreacted amino groups are to be expected that can react with fluorescamine to form the fluorophors. The slopes of the calibration plots from Figure 2 were proportional to the expected number of free amino groups per mole of insulin for various samples (Figure 3), indicating that the decrease in fluorescence correlated well with the conversion of free amino groups into intermediates that were unreactive towards fluorescamine.

This was an important finding since it established that the primary amines were indeed being converted into intermediates owing to DMMA and SPDP modifications. However this assay did not provide much information about the reaction site specificity of the modification agents. Hence, mass spectrometry analysis was performed on the insulin intermediates to confirm the modifications and to evaluate the specificity of the reagents for reaction with the primary amines.

Mass Spectrometry Analysis of the Insulin Modifications

Figure 4 shows the mass spectrum of unmodified bovine insulin in the negative ESI mode. The mass to charge ratio (m/z) for the −4 charge state is 1432.2 corresponding to the molecular

weight of 5732.8 Da. Figure 5 shows the ESI mass spectrum for DMMA-modified insulin sprayed from a 99/1/1 MeOH/H_2O/TEA solution. As seen from the mass spectrum, the 1:1 and 2:1 DMMA:insulin species are major products, and there is a minor amount of unreacted insulin apparent. Importantly, no peaks corresponding to the 3:1 derivative of insulin were found, supporting that non-specific modification of insulin did not occur and consistent with restriction of the modification sites to the two N-terminal amines (A- and B-chains). The ESI-mass spectrum of the SPDP- modified insulin intermediate is shown in Figure 6. The 2:1:1 DMMA:insulin:PDP species is identified at m/z 1535.6 and is shown to be the major reaction product from the mass spectrum.

To further pinpoint the sites of DMMA- and SPDP-modifications of insulin, ESI-MS was used to analyze the DMMA/SPDP- insulin product mixture after trypsin digestion. Figure 7 summarizes the potential sites of DMMA and SPDP modifications and the amide bonds expected to be cleaved by trypsin. Trypsin can cleave insulin at the amide bond between the B22Arg and B23Gly residues, yielding a 43 amino acid peptide (Ins-43) containing both the A- and B-chain N-terminals and a smaller eight amino acid peptide (Ins-8) containing the B-chain C-terminal of insulin. The trypsin cleavage site between B29Lys and B30Ala is not accessible since the normally protonated side-chain of Lys, which signals tryptic cleavage, is blocked by the PDP modification. The two resulting tryptic peptides were structurally characterized by tandem mass spectrometry. Figure 8 shows the ESI- mass spectrum of the Ins-43 tryptic peptide (+3 charge state). As expected, peaks corresponding to both 1:1 and 2:1 DMMA:In-43 were identified in the mass spectrum, but no peaks were found for the corresponding PDP attachment to Ins-43 which would be observed at m/z 1672.7, 1708.3 or 1744.0 depending on the degree of DMMA modification. This confirms that the PDP did not attach to the N-terminal primary amines of the A or B chains.

Collisional activated dissociation (CAD), a method used to provide structural information based on the fragmentation pattern of a selected ion in the gas phase, was used for further characterization of the smaller of the two tryptic peptides, (Ins-8). The ESI tandem mass spectrum for the eight amino acid peptide, Ins-8, is shown in Figure 9. The parent ion at m/z 565 corresponds to diprotonated Ins-8 with a single PDP modification and is consistent with the synthetic strategy outlined in Scheme 1B and the predicted product shown in Figure 7. An array of b and y sequence ions is observed which confirms the amino acid sequence of the Ins-8 species, and the sequence ions that are mass-shifted by 198 Da indicate the incorporation of the PDP group. From the series of fragments generated by CAD, two critical sequence ions were identified: y_4 (TPKA)-PDP and b_7 (G-F-FYTPK)-PDP (see inset of Figure 9), with each one containing the B-chain 29Lys residue. The m/z values of these two key sequence ions confirm the presence of PDP in both species and thus narrow the possible PDP modification sites to the residues B27Thr, B28Pro and B29Lys because these are the only amino acids common to the two sequence ions and the Ins-8 precursor. Further dissociation of the selected y_4 (TPKA)-PDP species via a second stage of CAD yields the y_2 (KA)-PDP ion at m/z 415.0 as shown in Figure 10, along with the dominant loss of the thiopyridyl moiety, 111 Da, from the PDP group of the y_4 (TPKA)-PDP parent ion which results in the abundant ion at m/z 502. The presence of y_2 (KA)-PDP species from this MS^3 experiment and the b_7 (GFFYTPK)-PDP species from the prior MS/MS experiment confirms B29Lys as the sole site of PDP modification because it is the only amino acid found in both species. All other peaks observed in these mass spectra support this modification site without contradiction.

The data from mass spectrometry and the fluorescamine assay together provide evidence that the SPDP modification is primarily at B29Lys and the DMMA modification sites are A1Gly and B1Phe. These are important results since improper modification may result in reduction or complete loss of insulin activity.

HPLC Analysis of the Insulin-Transferrin Conjugates

Figure 11 shows the HPLC chromatogram for insulin, transferrin and the purified conjugate. The insulin eluted from the C4 column due to the applied gradient with a retention time of approximately 3.4 min, where as the transferrin and the conjugates eluted at around 4.5 and 7.5 min respectively. More importantly, the chromatogram of the purified conjugate did not show any peak for transferrin or insulin (data not shown), indicating that the conjugate was essentially pure and did not contain either of the unmodified proteins.

Analysis of the Stability of Insulin-Transferrin Conjugates to Enzymatic Degradation

Intact insulin-transferrin conjugate has been shown to reach systemic circulation in diabetic rats (12). In these studies insulin and insulin transferrin conjugates were orally administered in diabetic rats in a 30 mg/mL $NaHCO_3$ solution to neutralize the gastric acidity and to protect digestion of proteins in the stomach (12). However there was no mechanism to reduce the degradation of insulin or the conjugate in the intestinal milieu. Even in the absence of such a mechanism, the conjugate was able to reach the systemic circulation without being completely degraded. This provides an indirect evidence of the increased stability of the conjugated insulin against tryptic and chymotryptic attack. However this improvement in the stability of the conjugate in enzyme solution has not been demonstrated experimentally.

Hence in this study, the degradation profiles of insulin, transferrin and the conjugate were evaluated in a solution containing trypsin based on HPLC analysis. As can be seen from Figure 12, most of the insulin degraded due to tryptic attack within 15–20 minutes of incubation, whereas transferrin was significantly more stable at the same enzyme concentrations. The conjugate degraded at a faster rate as compared to transferrin, but was more stable than the unmodified insulin. The degradation rate constants were calculated from the slopes of the semi-log plot of the percentage of initial protein remaining vs. time at early time points (Figure 13 and Table 1). The degradation rate constant calc ulated was 21.1×10^{-3} min^{-1} for insulin and 0.73×10^{-3} min^{-1} and 6.17×10^{-3} min^{-1} for transferrin and the conjugate, respectively. These data indicated that the stability of insulin was significantly increased due to its conjugation with transferrin. This may be one of the important factors contributing to the observed hypoglycemic effect of the orally administered conjugates (12). Although the reasons behind the observed increase in stability of the conjugate are not clearly understood, this may be due to the shielding effect of the transferrin which limits enzymatic degradation of insulin. Transferrin has been shown to be resistant to tryptic and chymotryptic digestion (13). Thus the large transferrin molecule may stabilize the conjugated insulin against enzymes by creating a hindrance for the enzymatic attack.

CONCLUSIONS

Insulin-transferrin conjugate was synthesized by site-specific modification of insulin and modification of transferrin by a heterobifunctional crosslinker. Proper modification of the insulin molecule, which is critical to its physiological function, was verified through fluorescence photometry and mass spectrometry. The conjugation resulted in a macromolecular heteroconjugate consisting of two insulin molecules and one transferrin molecule. Insulin in the conjugated form exhibited enhanced stability against proteolytic attack. The insulin-transferrin conjugates synthesized here exhibit beneficial characteristics for oral delivery applications. This system when used in conjunction with the complexation hydrogels developed in our laboratory may result in a efficacious system for oral delivery of insulin.

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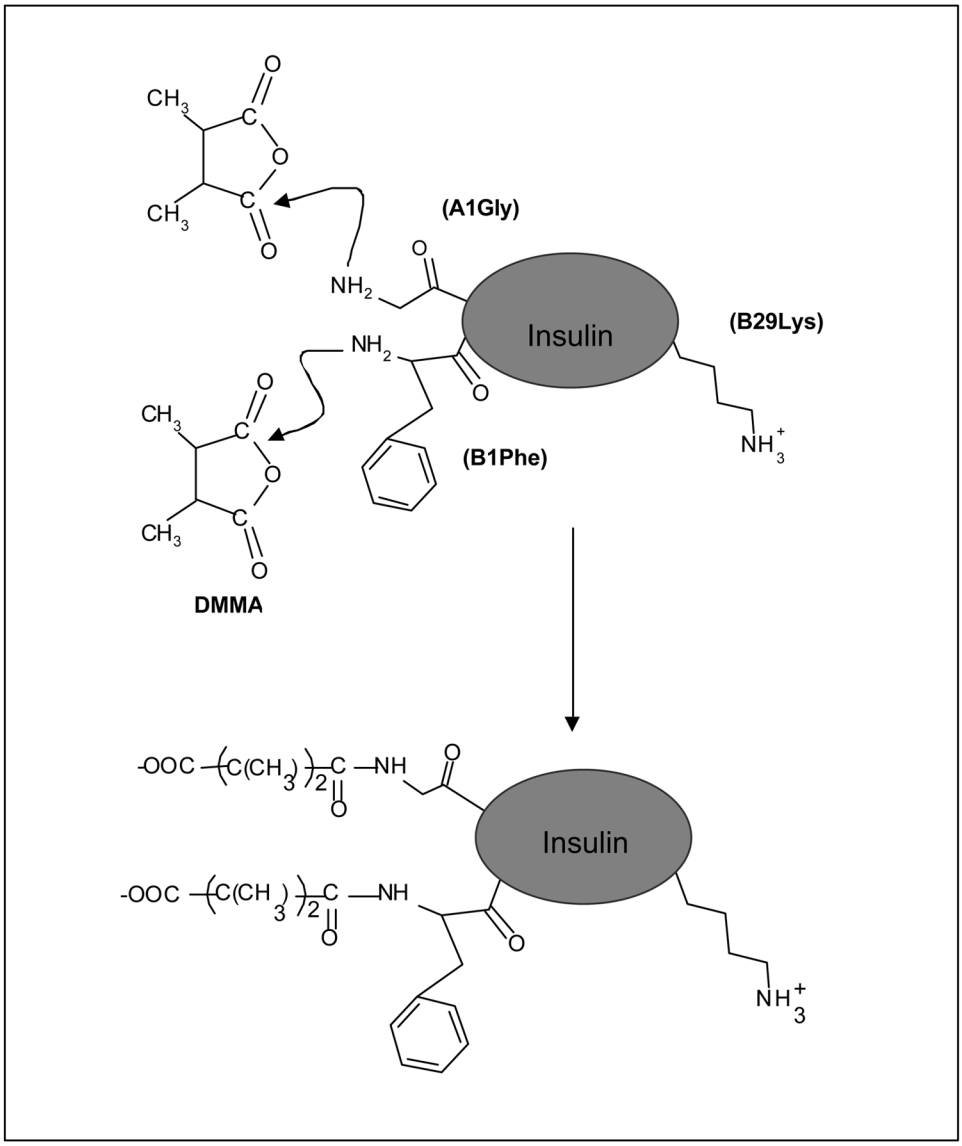

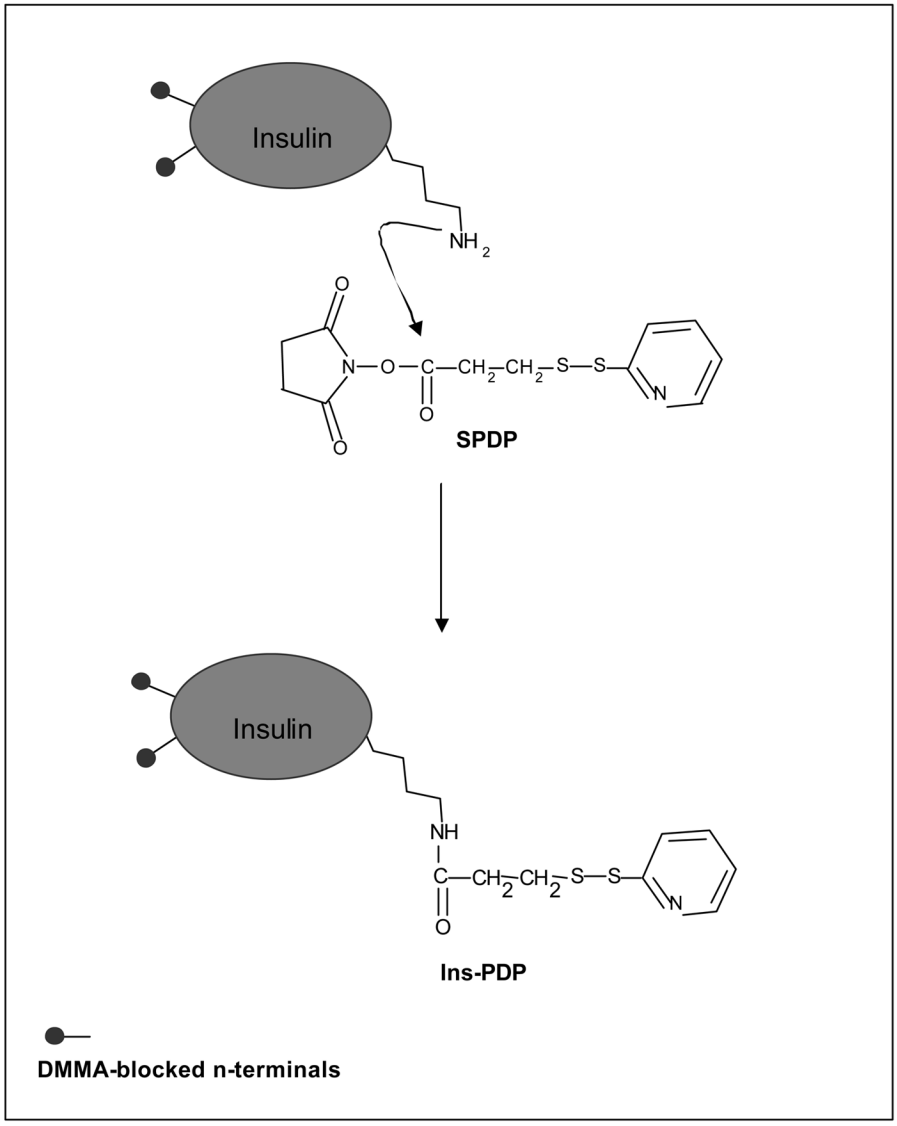

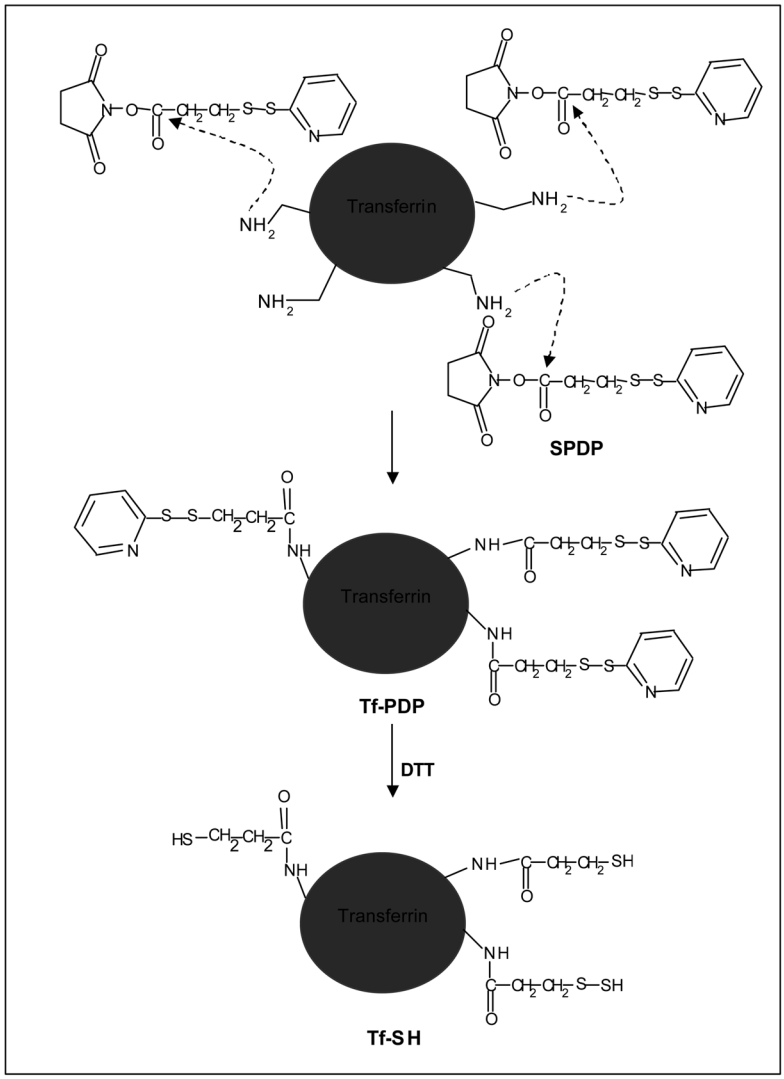

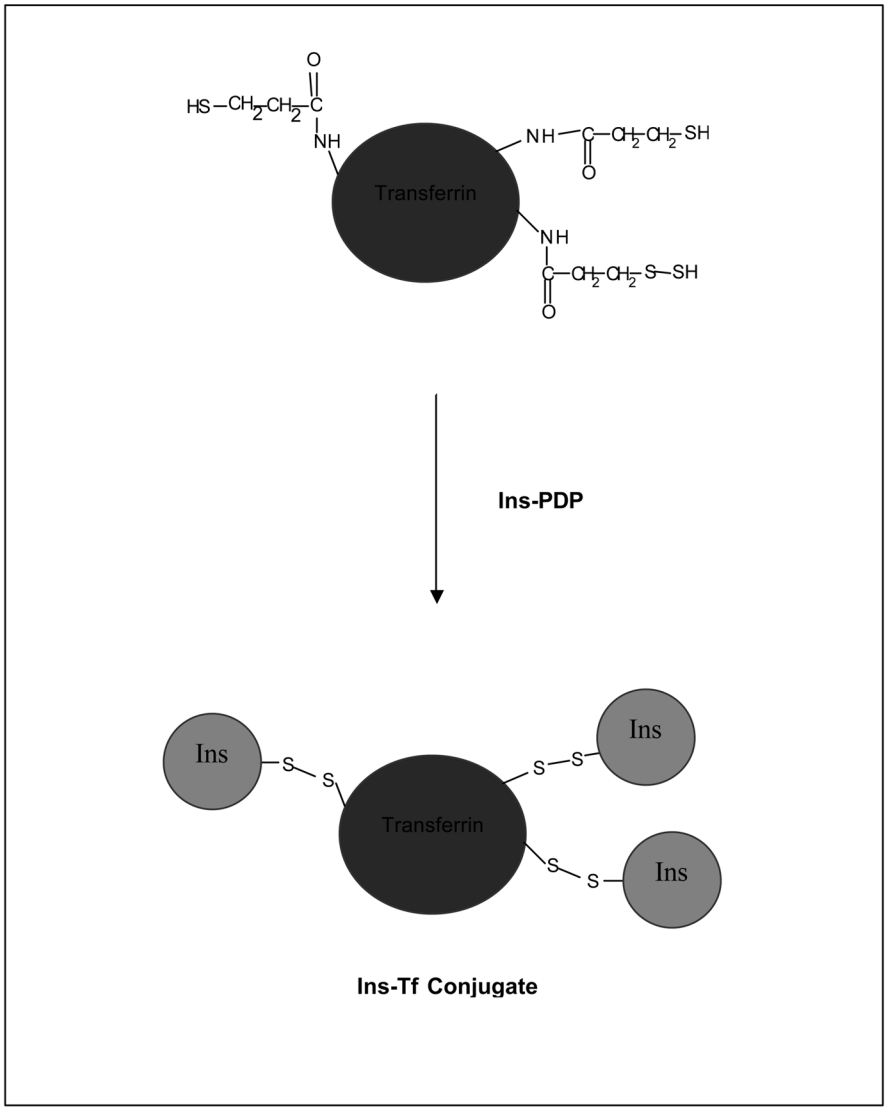

Figure 1.

Figure 1A. Conjugation reaction, step I (DMMA blocking of the -terminal amine groups). DMMA reaction with insulin at pH of 6.8–6.9. Only the primary amines at n-terminal are deprotonated and are able to react with DMMA. Most of the primary amines at B29 lysine residue are protonated and do not react with DMMA.

Figure 1B. Conjugation reaction, step II (SPDP reaction with DMMA-modified insulin). SPDP reacts with DMMA-modified insulin at pH 9.0 to form Insulin-PDP.

Figure 1C. Conjugation reaction, step III (SPDP modification of transferrin). SPDP reacts with transferrin at pH 7.0 to form Tf-PDP. The modification ratio can be controlled by altering the amount of SPDP reacted with transferrin. Purified Tf-PDP is then reacted with DTT to generate free thiol groups on transferrin (Tf-SH)

Figure 1D. Conjugation reaction, step IV (Ins-PDP reaction with Tf-SH). Purified Tf-SH reacts with Ins-PDP obtained from step II to form Ins -Tf conjugate.

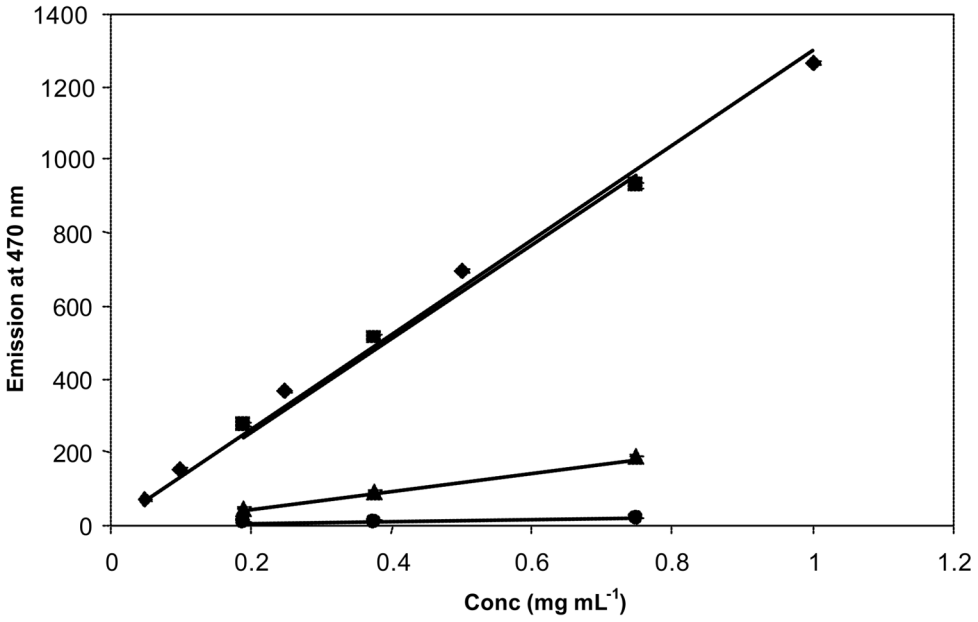

Figure 2.

Fluorescamine assay for determination of free amine groups of insulin. 50 μl of 1.08 mM fluorescamine in acetone was added to 150 μl protein samples (insulin standard (◆), unmodified insulin (■), DMMA-modified insulin (▲), or SPDP-modified insulin (●)) in a microplate reader. The fluorescence associated with the samples was measured with a microplate reader (Excitation at 390 nm and emission at 470 nm) (Error bars are smaller than the symbols; n=3).

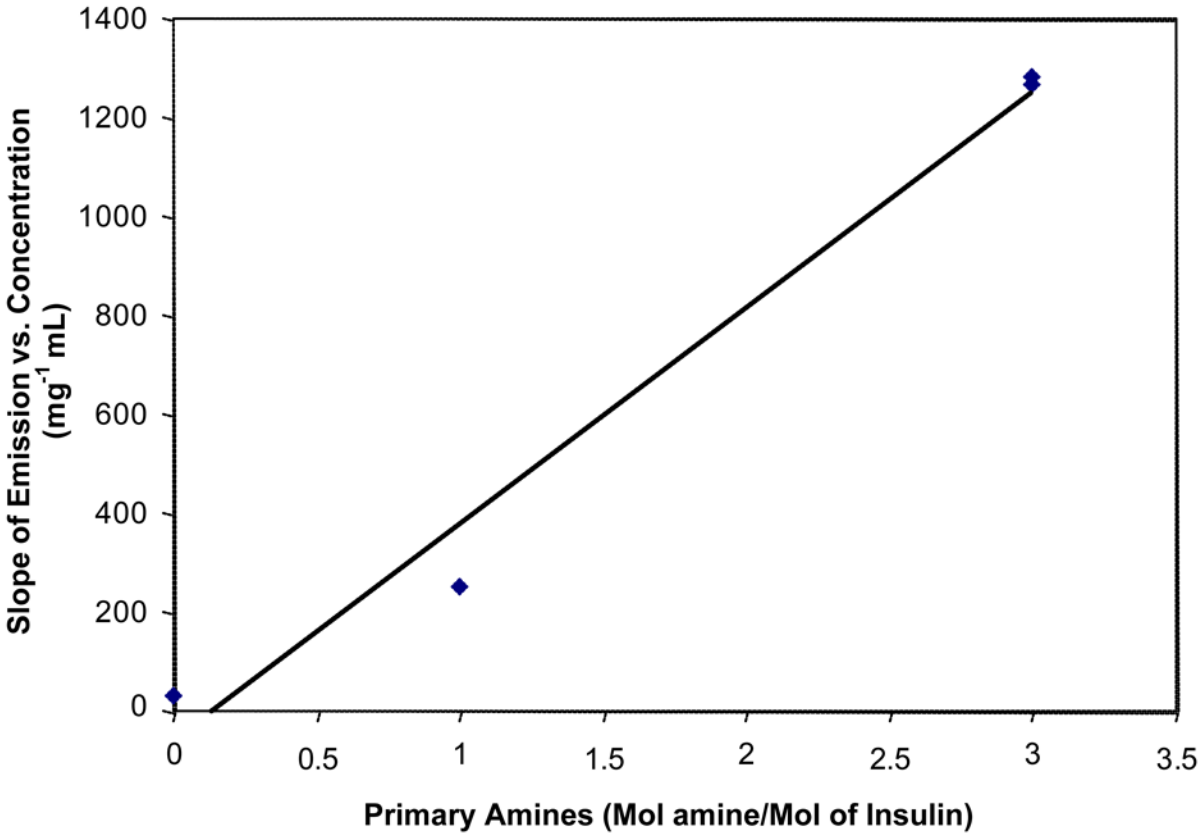

Figure 3.
Fluorescamine assay for determination of free amine groups of insulin. Slopes of the linear relationship between emission at 470 nm vs. concentration for various insulin samples (Figure 2) plotted against the expected number of moles of free amine groups per mole of insulin.

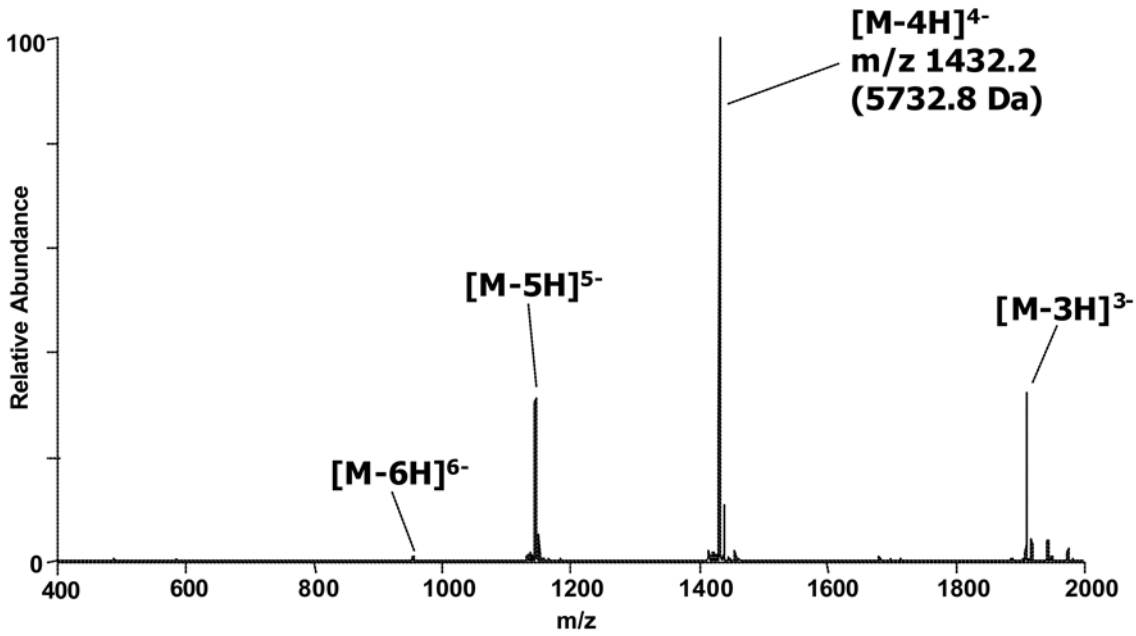

Figure 4.
ESI-mass spectrum of unmodified bovine insulin showing various charge states. The −4 charge state at m/z 1432.2 corresponds to a species with a molecular weight of 5732.8 Da. The spectrum was obtained in 99/1/1 MeOH/H_2O/TEA by volume.

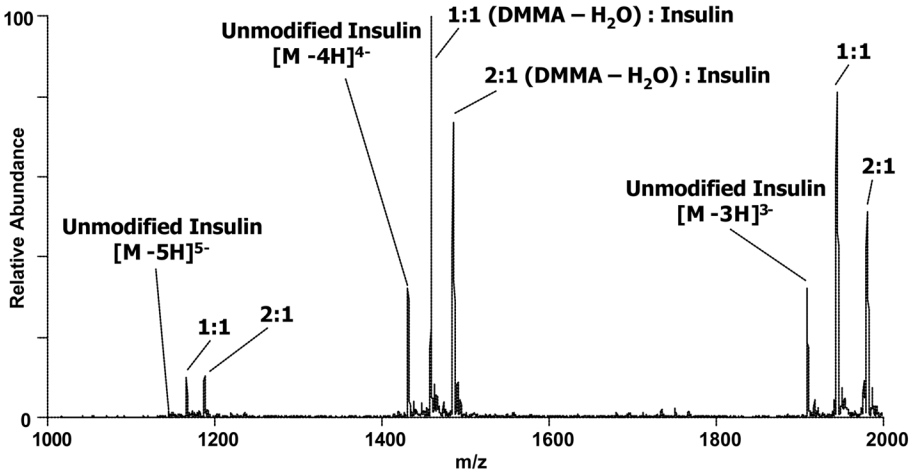

Figure 5.

ESI-mass spectrum of the DMMA-modified bovine insulin in 99/1/1 MeOH/H_2O/TEA by volume. The stoichiometry values (1:1 or 2:1) represent the number of (DMMA-H_2O) modifications per insulin molecule. The −4 charge state of the 2:1 (DMMA – H_2O):insulin species is observed at m/z 1486.3.

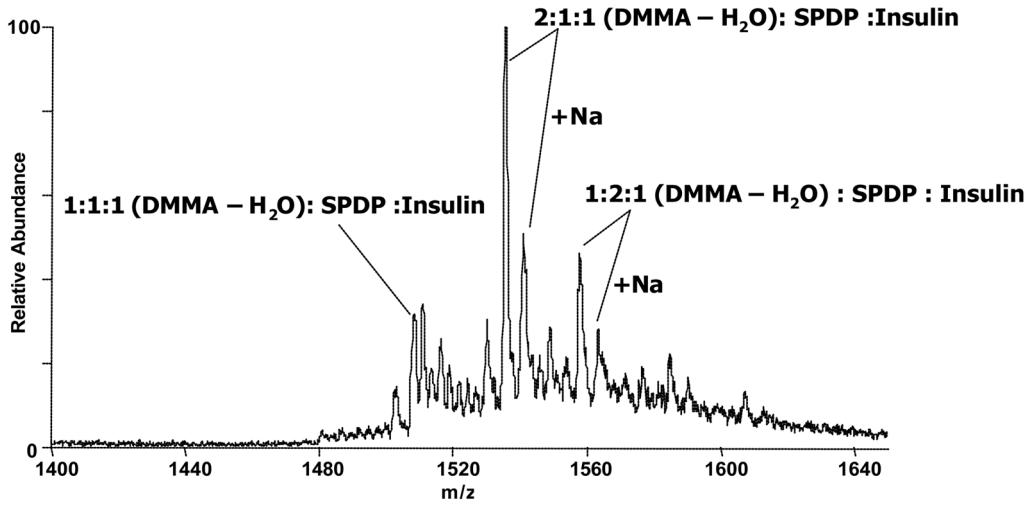

Figure 6.

ESI-mass spectrum of the SPDP-modified bovine insulin (−4 charge state) in 99/1/1 MeOH/H_2O/TEA by volume. The $[M - 4H]^{4-}$ and $[M – 5H + Na]^{4-}$ ions of the 2:1:1 DMMA:Insulin:PDP species are observed at m/z 1535.6 and 1540.9, respectively. Other minor peaks are attributed to various degrees of metal adduction from the ESI process.

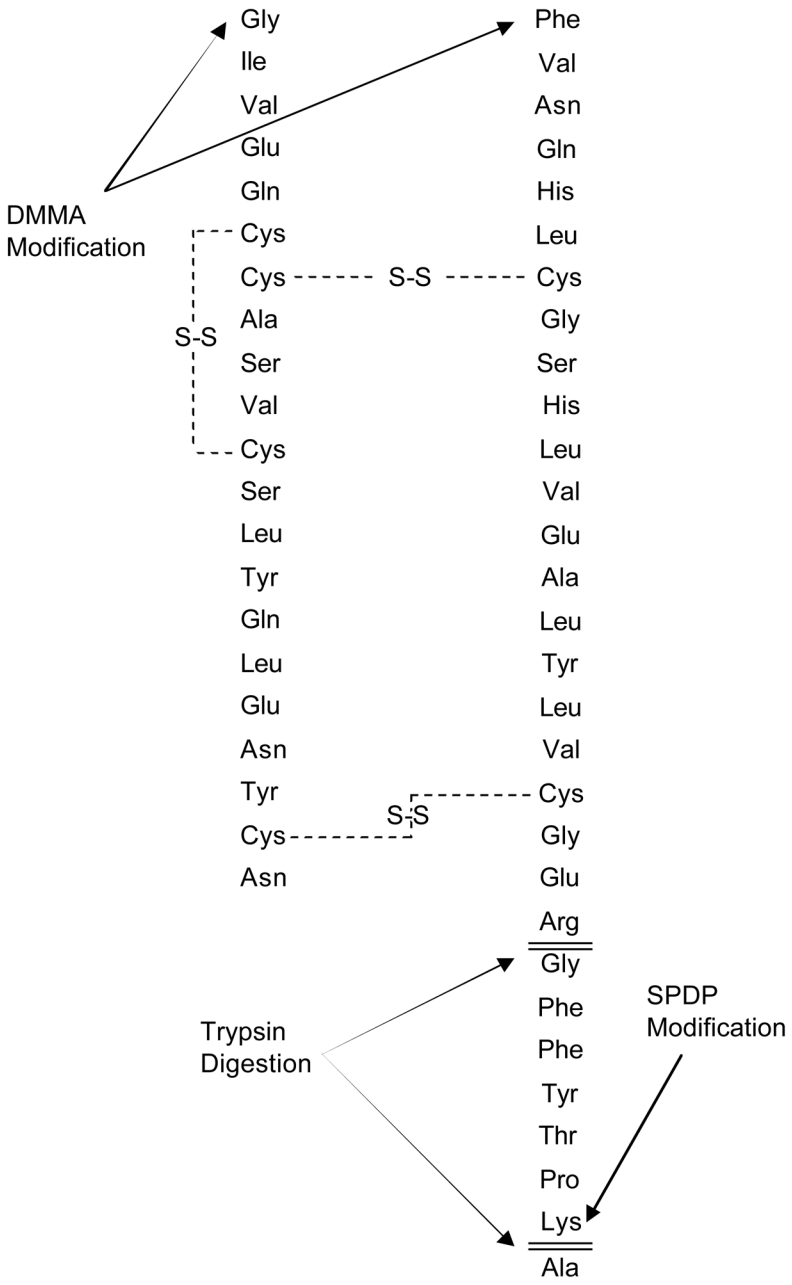

Figure 7.
Insulin primary structure showing modification sites of DMMA, SPDP and sites of trypsin digestion.

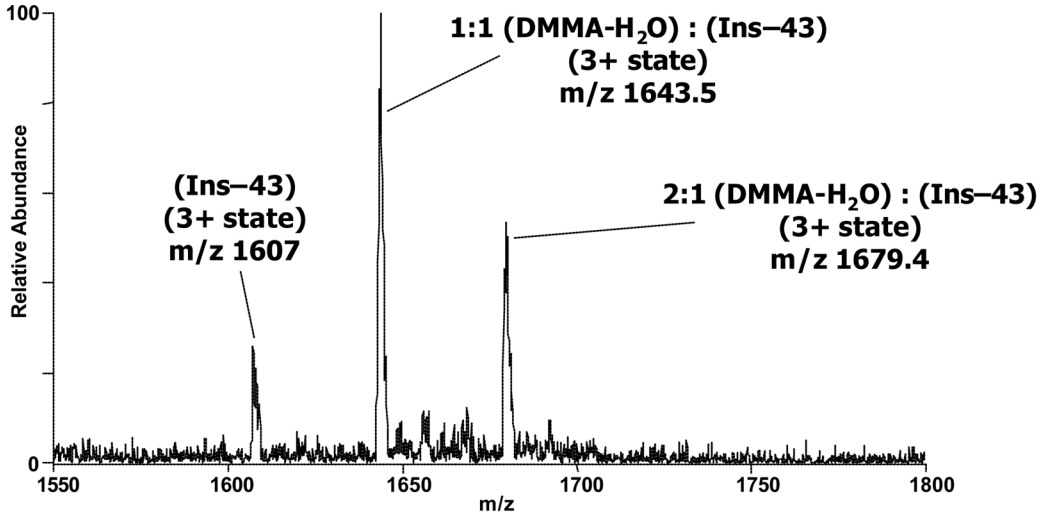

Figure 8.

Expansion of the ESI-mass spectrum of the +3 charge state region for the larger peptide formed after trypsin digestion of modified insulin (Ins-43). The spectrum was obtained in 50/50/1 H_2O/MeOH/HoAc by volume. No peak for the PDP derivative of Ins-43 was identified in the mass spectrum.

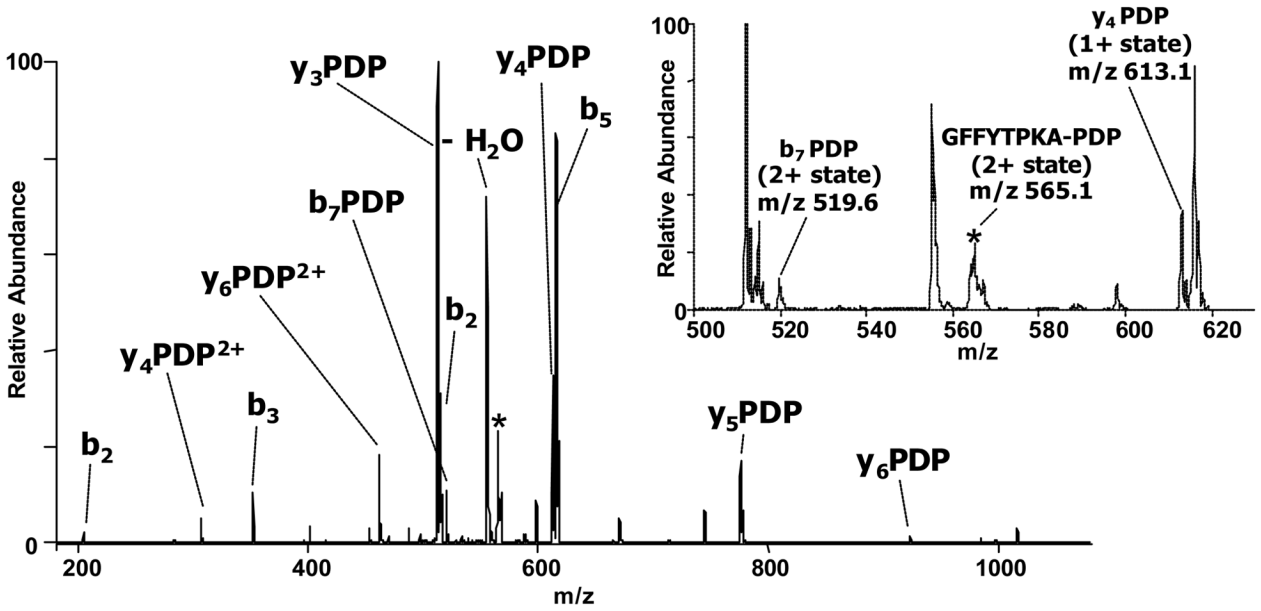

Figure 9.

ESI-MS/MS spectrum of the smaller peptide (m/z 565.1, (Ins -8)) formed after trypsin digestion of modified insulin. The insert shows the expanded region from the middle portion of the spectrum which contains two key diagnostic ions, $(b_7\text{-PDP})^{2+}$ at m/z 519.6, and $(y_4\text{-P DP})^{1+}$ at m/z 613.1.

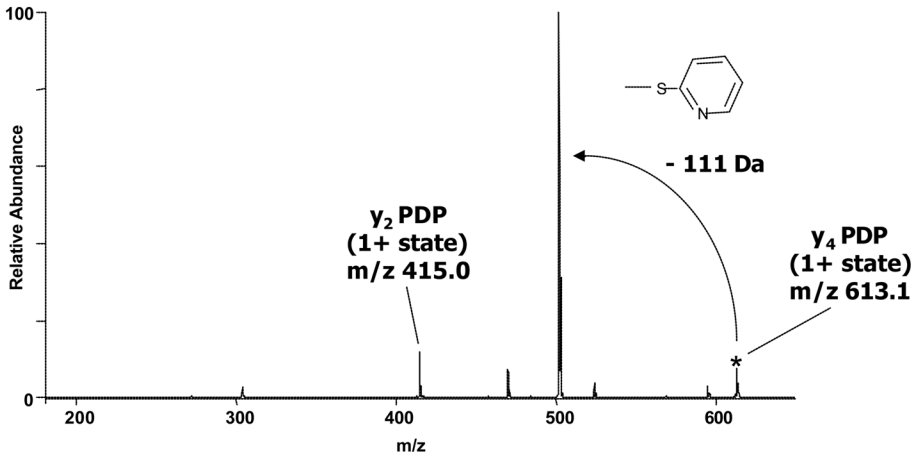

Figure 10.

ESI-MS^3 spectrum of the y_4-PDP peptide fragment at m/z 613.1. The y_2-PDP fragment is observed at m/z 415.0; this ion combined with the observation of b_7-PDP in Figure 9 provides confirmation of the B29Lys position as the specific site of modification. The major peak at m/z 502 is the loss of the thiopyridyl moiety from the PDP modification.

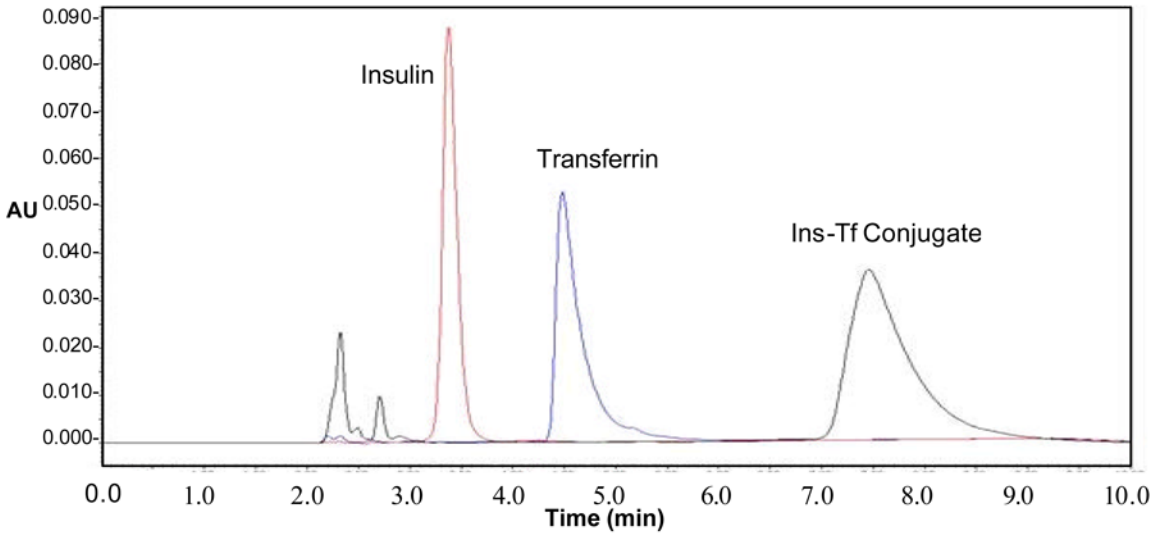

Figure 11.

HPLC chromatograms of insulin, transferrin and insulin-transferrin conjugate. The mobile phase for the analysis consisted of a linear gradient of 70% of solvent A to 40% of solvent A in 6 min. A Symmetry300™ C4 column (particle size 5 μm, 3.9mm i.d. ×150mm length) was used for the separation.

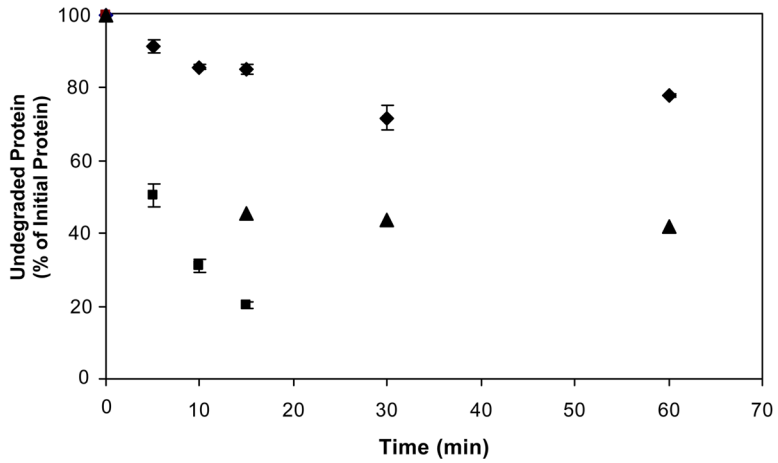

Figure 12.

Protein degradation profiles in the presence of trypsin. Initial protein concentration in the reaction was 1 mg/mL and the trypsin concentration was 3.2 mg/mL. The total reaction volume was 5 mL and the pH was 7.4. 50 μl samples were withdrawn at different time intervals and the trypsin activity in the samples was stopped by addition of equal volumes of 50% acetic acid solution. Insulin (■) and transferrin (◆) samples n=3; Conjugate (▲) sample n=1.

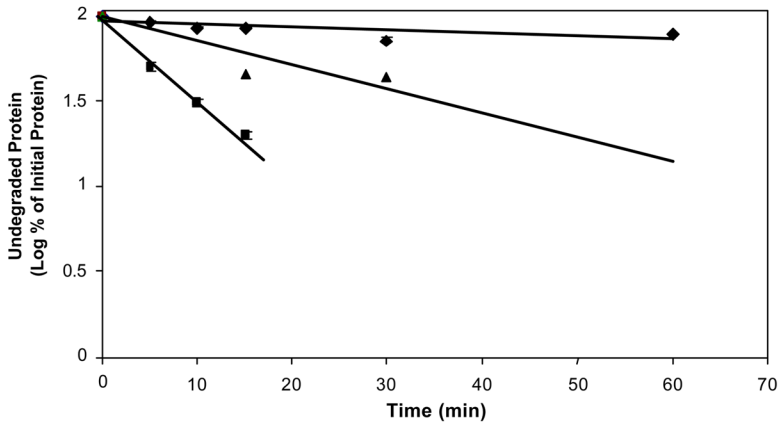

Figure 13.

Semi-log plot of % protein remaining vs. time (min). Initial protein concentration in the reaction was 1 mg/mL and the trypsin concentration was 3.2 mg/mL. The total reaction volume was 5 mL and the pH was 7.4. 50 μl samples were withdrawn at different time intervals and the trypsin activity in the samples was stopped by addition of equal volumes of 50% acetic acid solution. Insulin (■) and transferrin (◆) samples n=3; Conjugate (▲) sample n=1.

Table 1

Degradation rate constants for insulin, transferrin and conjugate in the presence of trypsin. Rate constants were calculated from the slopes of semi-log plot of % undegraded protein protein remaining vs. time at early time points (Figure 13).

Protein Sample	Slope (min^{-1})	Degradation rate constant (K) (min^{-1}) × 10^3
Insulin	−0.0486	21.10
Transferrin	−0.0017	0.73
Conjugate	−0.0142	6.17