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Does Epigallocatechin-3-Gallate-Insulin Complex Protect Human Insulin from Proteolytic Enzyme Action?

Antoine Al-Achi[1]* and Deepthi Kota[2]

[1]Campbell University College of Pharmacy and Health Sciences, P.O. Box 1090, Buies Creek, NC 27506, USA
[2]Novel Laboratories, 400 Campus Drive Somerset, NJ 08873, USA

Abstract

Insulin is a polypeptide hormone produced by the β cells present in Islets of Langerhans of the pancreas. Either failure to produce (type 1 diabetes) or utilize insulin (type 2 diabetes) causes diabetes mellitus. Insulin administration is used to treat type 1 diabetes. The common route for insulin administration is via subcutaneous injection. The oral insulin delivery has been proposed, however it suffers from poor bioavailability which is mainly due to the presence of proteolytic enzymes (pepsin, trypsin, and chymotrypsin) in the gastrointestinal (GI) tract. Protecting insulin from these enzymes when given orally might improve its bioavailability. In general, condensed tannins have been shown to reduce the activity of digestive enzymes. Epigallocatechin-3-gallate (EGCG) is the most abundant tannin component found in green tea. The present study investigated the ability of EGCG to protect insulin, through the formation of EGCG-insulin complex, from the proteolytic enzyme action by pepsin and trypsin/chymotrypsin, in vitro. The amount of insulin remaining in the presence and absence of EGCG following incubation with either simulated gastric fluid (SGF) containing pepsin or simulated intestinal fluid (SIF) containing trypsin/chymotrypsin at two different temperatures (25˚C and 37˚C) for 1 hour and 7 hours was determined using an HPLC technique. The results showed that the presence of proteolytic enzymes (pepsin or trypsin/chymotrypsin) and absence of EGCG in the sample negatively affected the stability of insulin in solution. In the presence of EGCG, insulin was partially protected from trypsin/chymotrypsin but it was not protected from the action of pepsin. Insulin degradation was more pronounced at 37˚C than that at 25˚C (p = 0.0188). The initial concentration of insulin present (10 IU/mL or 20 IU/mL) or the time of incubation (1 h vs. 7 h) had no influence on the stability of insulin in the sample (p = 0.2842 and p = 0.2114, respectively). In conclusion, EGCG was not able to protect insulin against the proteolytic activity of pepsin. However, EGCG was shown to have some protective effect on insulin against the degradative effect of trypsin/chymotrypsin at room temperature, *in vitro*. Furthermore, this protection was greatly weakened at 37˚C, which suggested that the protective action of EGCG would not be present *in vivo*.

Introduction

Diabetes mellitus is a disease characterized by disturbances in the regular metabolism of carbohydrates, proteins and fats. Glucose enters cells with the assistance of insulin, however in the case of diabetes mellitus the movement of glucose into the adipose and skeletal muscle cells is reduced and thereby results in the decreased levels of glycogen [1]. Insulin is a protein (polypeptide hormone) produced by the β cells of the pancreas and is chemically composed of two polypeptide chains connected through two intermolecular disulfide bridges. The two polypeptide chains are named chain A and chain B with 21 and 30 amino acids respectively [2]. The free glucose circulating in the blood enters into the liver, muscles, and adipose tissues through insulin by stimulating the enzymatic reactions at the insulin receptors on the cell membrane. Membrane phosphorylation occurs by the stimulation of an intrinsic tyrosine kinase of the insulin receptor. It results in an enhancement of cell membrane permeability to glucose which involves some series of intracellular events [3]. Insulin is commonly administered in the form of subcutaneous injection, but there are several drawbacks associated with it, such as patient incompliance, local discomfort, and occasional hyper-insulinemia because of overdose [4,5]. Alternative routes of insulin delivery have been proposed such as per-oral (enteric-gastrointestinal) route, oral-buccal and sublingual routes, rectal delivery, ocular and intravaginal routes, transdermal delivery, intranasal delivery, and pulmonary route [6,7]. Although the majority of these non-invasive routes have not produced acceptable safety or efficacy profiles [7], such as the case with polymer-encapsulated-insulin delivery [8], the nasal and oral routes remain the most promising for the insulin delivery [4].

Overall, the oral route is considered to be a patient-friendly mode of administration. However, protein administration suffers from poor bioavailability when given orally. Pharmaceutical scientists have adopted several strategies in order to improve on the bioavailability of orally administered insulin. Among these novel approaches were protecting insulin from the enzymatic degradation using anti-proteolytic agents; penetration enhancers to increase gastrointestinal absorption of insulin; improving the stability of insulin by making some chemical modification to it; enhancing the contact of drugs with the mucus lining of the GI tract using the bio adhesive delivery systems; and using various carrier systems like nanoparticles and microspheres to improve the insulin bioavailability [4]. Oral insulin is easy to administer, has better patient compliance, has low index of intrusion which results in glycemic control, and also lowers the diabetic complications [9]. High porto-systemic gradient can be obtained by oral insulin because it is delivered from the GI tract to the liver. It mimics in that way the natural pathway for insulin handling by the body [10].

*Corresponding author: Antoine Al-Achi, Associate Professor, Department of Pharmaceutical Sciences, Campbell University College of Pharmacy and Health Sciences, P.O. Box 1090, Buies Creek, NC 27506, USA, Tel: 910-893-170; E-mail: alachi@campbell.edu

Tannins are the polyphenolic secondary metabolites obtained from the higher group of plants which can be either galloyl esters or their derivatives [11]. These are the complex organic and non-nitrogenous plant products with astringent properties [12]. The studies conducted on experimental animals and on cell cultures using tannins have revealed that these compounds have various useful effects. According to these studies, it is believed that there are two different ways by which the tannins interact with proteins either by forming a non-absorbable complex structure or an absorbable complex with the proteins [13]. One of the important tannins known to man is Epigallocatechin-3-gallate (EGCG). It is a tannin component found in green tea (*Camellia sinensis*). In experimental animals, EGCG acted in a similar manner to insulin in reducing blood glucose in mammals through phosphorylation induction of insulin-sensitive residues on the transcription factor FOXO1a [14]. Previous studies have shown that the condensed tannins have decreased the activity of digestive enzymes obtained from various parts of rats and chicken intestinal tracts. Moreover, the activities of trypsin and α-amylase were reduced in various parts of the small intestine of rats after feeding them with the tannin-rich extracts obtained from different fodder plants [15] which may be associated with the ability of EGCG to bind with cross-beta sheet aggregation intermediates of proteins [16]. Moreover, this inhibitory effect of EGCG on proteolytic enzymes (e.g., trypsin) was shown to be due to binding of EGCG to the enzymes through hydrogen bound formation [17], and this binding capability may be reversed by salivary proline-rich proteins [18]. Although EGCG had an inhibitory action on trypsin and chymotrypsin, it had no effect on the action of pepsin [18,19].

EGCG was shown to be capable of binding to insulin by hydrophobic interactions and by the formation of hydrogen bounds [20,21]. The present study investigated the ability of EGCG to protect insulin, through the formation of EGCG-insulin complex, from the proteolytic enzyme action by pepsin and trypsin/chymotrypsin, *in vitro*.

Methodology

Materials

Table 1 and Table 2 shows the list of materials and equipment used, respectively.

Methods

Ultraviolet (UV) spectrophotometry: Using a saturated solution of EGCG [the maximum solubility of EGCG in water is 25 mg per 1 mL (0.055 M) at room temperature [6]], the detection wavelengths of EGCG were found to be at 206 nm and 274 nm.

High Performance Liquid Chromatography Method (HPLC) for human insulin: The parameters used on the HPLC system for the quantification of the human insulin are shown in Table 3. A high linear correlation was observed for the calibration curve (1 IU/mL to 100 IU/mL; $R^2 = 0.9993$; $p < 0.0001$). No interference with insulin peak was detected in the presence of proteolytic enzymes or EGCG. A peak for EGCG was also detected with the same HPLC conditions, except for using 274 nm as a detection wavelength. No interference with the insulin peak was observed when EGCG and/or proteolytic enzymes were present in solution (Table 3).

Determination of binding efficiency of EGCG and insulin: Insulin solutions (1 IU/mL to 40 IU/mL; 0.006 mmol/L to 0.24 mmol/L) were prepared in duplicate and mixed with an equal volume of EGCG solution (25 mg/mL; 0.055 M). The mixtures were kept undisturbed for

Chemical	Manufacturer
(−)- Epigallocatechin gallate (MW = 458.37 Daltons)	Sigma-Aldrich
Humulin-R (100 IU/mL)- Regular insulin human injection, USP (rDNA origin) [1 IU/mL = 0.0347 mg/mL]	Eli-Lilly
Acetonitrile	Fischer Scientific
Trifluoroacetic acid	Fischer Scientific
Isopropyl alcohol	EMD
Potassium phosphate monobasic	Sigma Life Sciences
Sodium chloride	Sigma-Aldrich
Porcine trypsin (1:250), Gamma Irradiated	Sigma-Aldrich
Pepsin from porcine gastric mucosa (≥ 250 units/mg solid)	Sigma Life Sciences
α- chymotrypsin (EC 3.4.21.1, Type II: From bovine pancreas)	Sigma Life Sciences
Sodium hydroxide	Sigma-Aldrich
pH 7.00 buffer Contents: Orion 910107	ThermoElectron Corporation
pH 4.01 buffer Contents: Orion 910104	ThermoElectron Corporation
Hydrochloric acid (HCl) 12 N	Sigma-Aldrich

Table 1: List of materials used.

Equipment and Model	Manufacturer
Microbalance	Sigma Life Sciences
UV-Vis spectrophotometer	Agilent
Spectrophotometer cell (Cuvette) Capacity: 0.7 mL, 10 mm light path	VWR International
Micropipette Capacity: 5000 µL	Gilson
Volumetric flasks Capacity: 5 mL, 10 mL, 25 mL, and 100 mL	Pyrex
Transfer pipets (Disposable Polyethylene)	Fischer Scientific
Vortex machine (Model: Genie 2)	Fischer Scientific
HPLC clear shell vials w/plugs, Capacity: 1 mL	VWR International
Centrifuge	Sorvall Instruments
Centrifuge rotor Fixed angle: SS-34 05 20,000 rpm	Sorvall Instruments
Weighing balance Model: AE163	Mettler
Micropipettes, Capacity: 10 µL, 20 µL, 100 µL, 1000 µL, 5000 µL	Gilson
Micropipette tips Capacity: 100 µL, 1000 µL	Biohit
HPLC (LC-10AD)	Shimadzu
UV-Vis detector (Model:SPD-10A)	Shimadzu
Degasser (DGV-14A)	Shimadzu
Peak Simple Chromatography Data System (6 channel USP port)	SRI Instruments
Autosampler	Waters
C_4 protein column Dimensions: 15 cm × 4.6 mm	Vydac
Syringes (TB syringes, slip tip with Intradermal Bevel Needle) Capacity: 1 mL	EXEL International
Millex 0.22 µm Syringe-driven Filter Unit (PVDF filters)	Millipore
pH meter (benchtop) Model: Orion 3 star	Thermo Electron Corporation
Syringes, Capacity: 10 mL disposable, Model: 10 mL Luer slip tip	EXEL International

Table-2: List of equipment used.

Parameter	Value
Injection Volume	15 µL
Flow Rate	2.0 mL/min
Wavelength	215 nm
Run Time	20 minutes
Column	Vydac C_4 protein column (15 cm × 4.6 mm)
Mobile phase	
Component A	Acetonitrile (10-60%) with 0.05% Trifluoroacetic acid
Component B	Water (90-40%) with 0.05% Trifluoroacetic acid
Method used	Gradient elution

Table-3: HPLC parameters for the quantification of insulin.

one hour at room temperature, to allow sufficient complex formation. After one hour incubation, the mixtures were centrifuged at a speed of 12,000 rpm for 20 minutes at 25°C. Following centrifugation, the supernatant was collected from each tube and was analyzed for insulin using HPLC.

pH adjustment studies: The purpose of these experiments was to determine the volume of either 50% w/v NaOH or 12 N HCl needed to affect a change in pH of solution containing proteolytic enzymes for deactivating pepsin or trypsin/chymotrypsin, respectively. For proper proteolytic action, pepsin requires an acidic pH and trypsin/chymotrypsin combination requires a neutral to basic pH. Thus, by changing the pH of the solution containing pepsin to a basic pH or that containing trypsin/chymotrypsin to an acidic pH would immediately halt the enzymatic action on insulin by the enzymes [23]. Simulated Gastric Fluid (SGF) was prepared by dissolving 0.0225 g of sodium chloride and 0.0332 g of pepsin in 7-mL of distilled water. To the mixture, 0.07 mL of 12 N HCl was added using a micropipette and the final volume was made to 10 mL using distilled water. The pH of the final solution was adjusted to 1.2 using 15 µL of 12N HCl. Simulated Intestinal Fluid (SIF) was prepared by dissolving 0.0679 g of monobasic potassium phosphate, 0.0509g of trypsin, and 0.0502 g of chymotrypsin in 1.9 mL of 0.2 N sodium hydroxide solution and the final volume was made to 10 mL using distilled water. The pH of the above solution was adjusted to 7.6 using 50% w/v NaOH solution. Solutions containing EGCG-insulin (10 IU/mL or 20 IU/mL; 0.06 mmol/L or 0.12 mmol/L) in the presence of SGF or SIF (Test Group) were treated with 50% w/v NaOH or 12 N HCl, respectively in order to halt the degradative action of the enzymes on insulin. In addition solutions containing only insulin (10 IU/mL or 20 IU/mL) with SGF or SIF (Control I) or only EGCG (0.055 M) with SGF or SIF (Control II) were also adjusted to basic pH or acidic pH with 50% NaOH or 12 N HCl, respectively. Samples were treated for either 1 h or 7 h under aforementioned conditions prior to adding the 50% NaOH or 12 N HCl. The volume of 50% w/v NaOH and 12 N HCl needed to affect this change in pH were recorded (Table 4, Table 5). For all the samples prepared, the concentration units listed for the various components reflected those found prior to mixing with SGF or SIF solutions. SGF and SIF solutions were mixed with solutions containing only insulin, only EGCG, or only EGCG-insulin complex in a ratio of 1:2 (v/v) (sample:SGF or sample:SIF) (actual volumes were 100 µL of sample mixed with 200 µL of proteolytic enzyme solution).

Effect of proteolytic enzymes on the stability of insulin in the presence and absence of aqueous EGCG solution: An experiment was conducted to determine the effect of proteolytic enzymes on the stability of insulin in the presence (0.055 M) and absence of EGCG solution. Solutions containing EGCG-insulin (10 IU/mL or 20 IU/mL; 0.06 mmol/L or 0.12 mmol/L) in the presence of SGF or SIF (Test Group) and solutions containing only insulin (10 IU/mL or 20 IU/mL) with SGF or SIF (Control I) or only EGCG (0.055 M) with SGF or SIF (Control II) were prepared (Control II samples were used to ascertain that the conditions of incubation used did not interfere with the insulin peak on HPLC assay. As stated above, no interference with the insulin peak was observed when EGCG and/or proteolytic enzymes were present in solution). Samples were treated for either 1 h or 7 h in triplicates and kept under 25°C or 37°C (insulin was stable under those conditions if proteolytic enzymes were not added to solution). After pH adjustment to halt the action of proteolytic enzymes on insulin degradation as specified above, the samples were filtered and analyzed on HPLC for their content of insulin. For all the samples prepared, the concentration units listed for the various components reflected those found prior to mixing with SGF or SIF solutions. SGF and SIF solutions were mixed with solutions containing only insulin, only EGCG, or only EGCG-insulin complex in a ratio of 1:2 (v/v) (sample: SGF or sample: SIF) (actual volumes were 100 µL of sample mixed with 200 µL of proteolytic enzyme solution).

Statistical analysis: JMP® Statistical Discovery Software (SAS Institute, Cary, NC) was used for the statistical analysis. A multifactorial analysis of variance method (MANOVA) was used to test the difference in insulin content (the dependent variable) remaining following the treatment with the enzymes in the presence of EGCG. The independent variables were the initial concentration of insulin present (10 IU/mL and 20 IU/mL), temperature (25°C and 37°C), and time of exposure to enzymes (1 h and 7 h). A p value of less than 5% was considered significant.
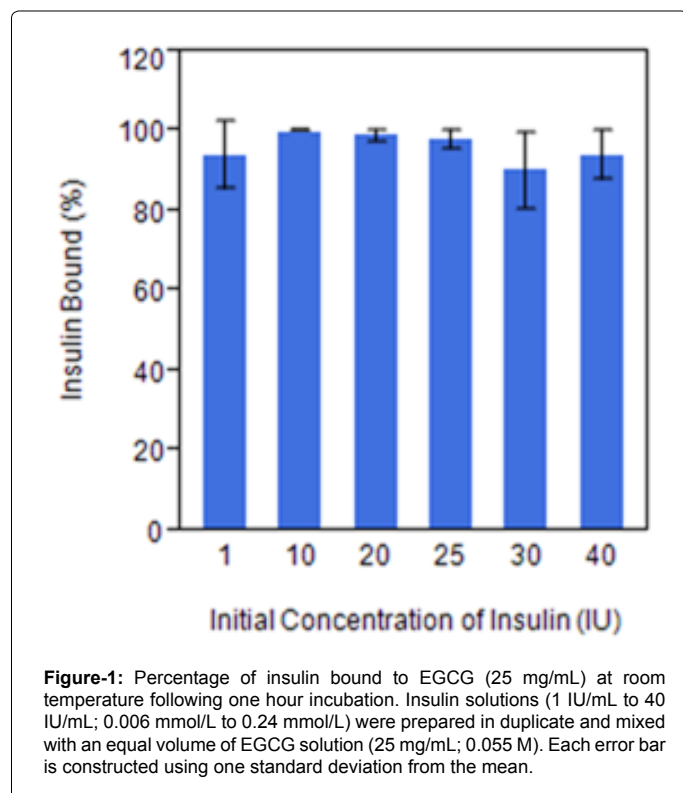
Results

The percentage of insulin bound to ECGC following incubation at 25°C for 1 hour is shown in Figure 1.

The volume (µL) of 50% NaOH or 12 N HCl needed to affect a change in pH are shown in Table 4 and Table 5, respectively. The effect of proteolytic enzymes on insulin (10 IU/mL or 20 IU/mL; 0.06 mmol/L or 0.12 mmol/L) degradation in the presence or absence of ECGC (25 mg/mL; 0.055 M) at different temperatures (25°C and 37°C) for 1 h or 7 h incubation periods is shown in Figure 2.

Discussion

For the initial concentrations of insulin used in this study (1 IU/mL to 40 IU/mL; 0.006 mmol/L to 0.24 mmol/L), almost all the insulin present in solution formed a complex with EGCG (mean ± S.D. 95.9% ± 5.6%; n = 12; 95% CI = 92.3% - 99.5%) (Figure 1) Wang et al. [24] have shown that EGCG directly bound to insulin primarily via hydrogen bond formation, and that the binding was independent of pH and temperature. In addition to hydrogen bound formation, EGCG-insulin complex was also held together by hydrophobic interactions [20,21]. The hydrogen bounds were expected to form between the hydroxyl groups of EGCG and certain currently undefined amino acid residues on insulin chain [18]. Insulin was quickly and completely degraded by SGF (pepsin) with or without EGCG being present in the solution (Figure 2). This agrees with the results obtained from a previous study where the proteolytic action of pepsin (SGF) on free insulin was completed within one minute of incubation with the enzyme [2]. Also, EGCG was shown to be unable to halt the enzymatic activity of pepsin, and in some cases even enhanced the proteolytic action of pepsin

Figure-1: Percentage of insulin bound to EGCG (25 mg/mL) at room temperature following one hour incubation. Insulin solutions (1 IU/mL to 40 IU/mL; 0.006 mmol/L to 0.24 mmol/L) were prepared in duplicate and mixed with an equal volume of EGCG solution (25 mg/mL; 0.055 M). Each error bar is constructed using one standard deviation from the mean.

[19]. Naz et al. [18] have shown that EGCG inhibited several digestive enzymes in the following descending order -amylase > chymotrypsin > trypsin > lactase; negligible or no inhibition of pepsin by EGCG was noted in these experiments.

The destruction of free insulin by trypsin/chymotrypsin (SIF) in the absence of an enzyme inhibitor was found to follow a first-order type reaction with a first-order degradation rate constant of 0.069 min^{-1} at 37°C, *in vitro* [23]. A multifactorial analysis of variance test (MANOVA) was performed on the data obtained from the insulin degradation experiments with SIF (trypsin/chymotrypsin) and in the presence of EGCG-insulin complex. [In the absence of EGCG, all of the insulin was degraded by trypsin/chymotrypsin (Figure 2)]. Based on the analysis of the results (Figure 2), the temperature was the only factor that significantly (p = 0.0188) affected the amount of insulin remaining in solution following treatment with trypsin/chymotrypsin (Figure 3).

The effect of the proteolytic enzymes on insulin was halted at the end of the experiment [23] by changing the pH of the sample from (average ± S.D.; n = 20) 2.58 ± 0.62 to 8.06 ± 0.57 in the case of pepsin, and from 7.64 ± 0.48 to 1.47 0.73 for trypsin/chymotrypsin samples (Tables 4 and 5). Under those pH conditions, free insulin existed as a monomer (pH ~ 2.0) or as a dimer (pH ~ 7.4) in solution. Moreover, the presence of EGCG in solutions containing insulin was shown to prevent insulin aggregation to some extent; this aggregation prevention reached its maximum point at insulin concentration in the range of 0.1-0.2 mmol/L [24] (in the present study, the insulin concentration was 0.06 mmol/L or 0.12 mmol/L). Samples containing EGCG-insulin

S.No	Conc. of Insulin added (IU/mL)	Sample	Proteolytic enzymes added	Incubation period (h)	Temperature (°C)	Initial pH	Volume of 50% w/v NaOH added (µL)	Final pH
1	10	Control I (10 IU/mL)	SGF	1	25	1.77	3	8.12
2	10		SGF	7	25	2.20	2	8.64
3	10		SGF	1	37	1.67	2	7.86
4	10		SGF	7	37	2.60	2	7.62
5	0	Control II (EGCG 0.055 M)	SGF	1	25	2.60	2	7.91
6	0		SGF	7	25	2.63	2	8.62
7	0		SGF	1	37	2.52	2	9.82
8	0		SGF	7	37	3.24	2	8.12
9	10	Test group (EGCG-insulin complex)	SGF	1	25	2.56	2	7.47
10	10		SGF	7	25	2.33	2	7.71
11	10		SGF	1	37	2.91	2	7.96
12	10		SGF	7	37	2.39	2	7.42
13	20	Control I (20 IU/mL)	SGF	1	25	2.45	2	7.57
14	20		SGF	7	25	2.41	2	8.04
15	20		SGF	1	37	2.03	2	8.24
16	20		SGF	7	37	4.72	2	7.62
17	20	Test group (EGCG-insulin complex)	SGF	1	25	2.69	2	7.44
18	20		SGF	7	25	2.82	2	8.64
19	20		SGF	1	37	2.60	3	8.06
20	20		SGF	7	37	2.39	4	8.34
					Average ± SD	2.58 ± 0.62	2.2 ± 0.5 µL	8.06 ± 0.57

Table-4: Volumes of 50% w/v NaOH used for pH adjustment of the samples.

S.No	Conc. of Insulin added (IU/mL)	Sample	Proteolytic enzymes	Incubation period (h)	Temperature (˚C)	Initial pH	Volume of 12 N HCl added (µL)	Final pH
1	10	Control I (10 IU/mL)	SIF	1	25	6.81	2	1.54
2	10		SIF	7	25	7.48	6	1.40
3	10		SIF	1	37	7.06	2	1.54
4	10		SIF	7	37	7.85	9	1.28
5	0	Control II (EGCG 0.055 M)	SIF	1	25	8.08	6	1.22
6	0		SIF	7	25	8.12	6	1.24
7	0		SIF	1	37	8.61	6	1.25
8	0		SIF	7	37	7.74	7	1.22
9	10	Test group (EGCG-insulin complex)	SIF	1	25	7.92	8	1.45
10	10		SIF	7	25	6.68	7	1.24
11	10		SIF	1	37	8.22	6	1.28
12	10		SIF	7	37	7.62	6	1.24
13	20	Control I (20 IU/mL)	SIF	1	25	8.12	6	1.32
14	20		SIF	7	25	7.58	5	1.28
15	20		SIF	1	37	7.52	5	1.30
16	20		SIF	7	37	7.52	4	1.33
17	20	Test group (EGCG-insulin complex)	SIF	1	25	7.70	8	1.31
18	20		SIF	7	25	7.07	5	1.25
19	20		SIF	1	37	7.56	5	1.36
20	20		SIF	7	37	7.49	3	1.25
					Average ± SD	7.64 ± 0.48	5.6 ± 1.8 µL	1.47 ± 0.73

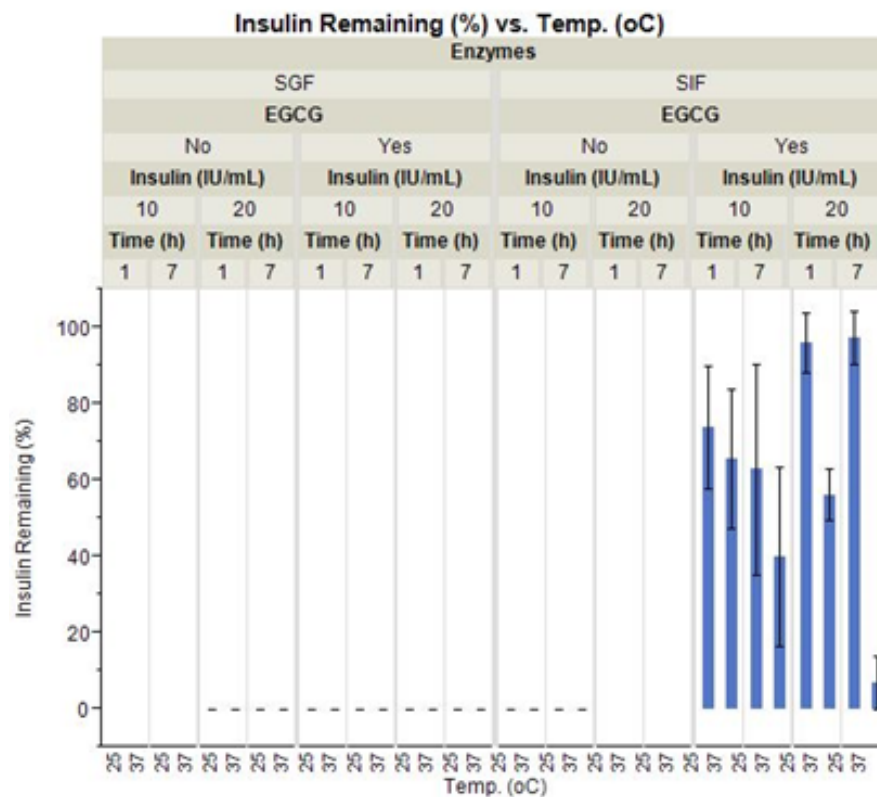**Table-5:** Volumes of 12 N HCl used for pH adjustment of the samples.

Figure-2: Percentage of insulin remaining after treatment with proteolytic enzymes (SGF = pepsin; SIF = trypsin/chymotrypsin) incubated at either 25˚C or 37˚C for 1 h or 7 h in the presence or absence of EGCG (25 mg/mL; 0.055 M). Initial concentration of insulin was 10 IU/mL or 20 IU/mL (0.06 mmol/L or 0.12 mmol/L). Each error bar was constructed using 1 standard error from the mean (n=6).
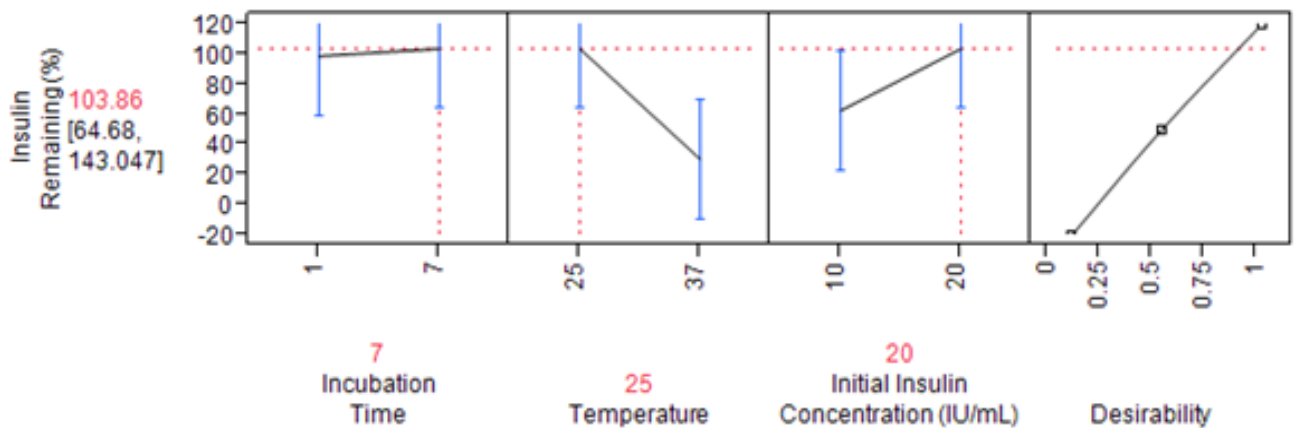
Figure-3: Profiler for the input factors and their values to produce a maximized value (by maximizing the desirability function) in the outcome: Proteolytic enzyme = SIF (trypsin/chymotrypsin), incubation time = 1 or 7 hours (p = 0.2114), temperature = 25°C or 37°C (p = 0.0188), and initial concentration of insulin = 10 or 20 IU/mL (p = 0.2842). Given incubation time of 7 hours, a temperature of 25°C, and an initial concentration of insulin of 20 IU/mL, the predicted percentage of insulin remaining was 103.86% (95% CI = 64.68% to 143.05%).
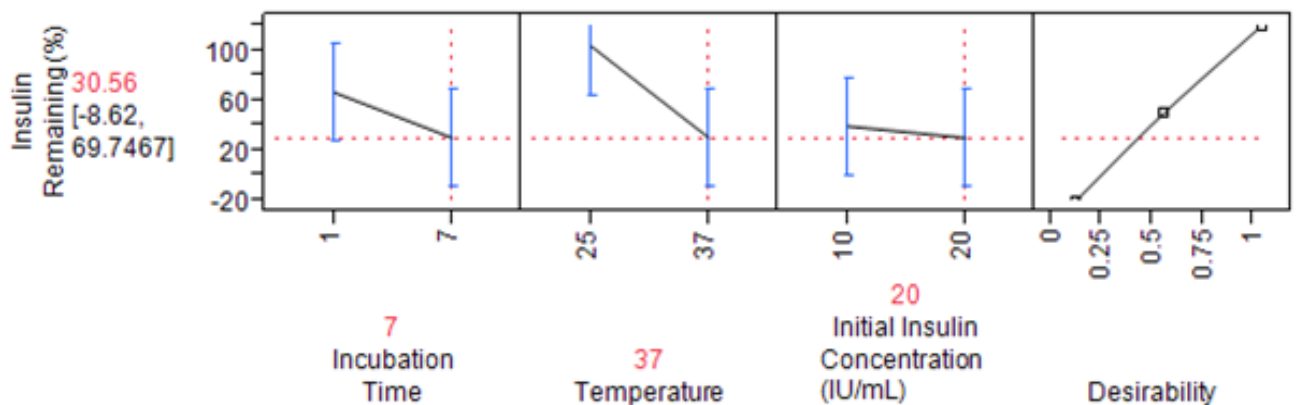

Figure-4: Profiler for the input factors and their collective effects on the outcome: Proteolytic enzyme = SIF (trypsin/chymotrypsin), incubation time = 7 hours, temperature = 37°C, and initial concentration of insulin = 20 IU/mL. Given these conditions, the predicted percentage of insulin remaining was 30.56% [95% CI = -8.62% to 69.75%].

complex and treated with trypsin/chymotrypsin had more pronounced Insulin degradation at 37°C than that at 25°C (p = 0.0188). On the other hand, the initial concentration of insulin present (10 IU/mL or 20 IU/mL) or the time of incubation (1 h vs. 7 h) had no influence on the stability of insulin in the sample (p = 0.2842 and p = 0.3012, respectively) (Figure-3). EGCG demonstrated no protective action on insulin in the presence of pepsin (SGF) under the experimental conditions, while it protected insulin to a certain degree when the hormone was incubated with SIF (trypsin/chymotrypsin) (Figure-2). This perhaps was due to the difference between pepsin and trypsin/chymotrypsin action on insulin. Human insulin is composed of 51 amino acids forming two chains (A and B). Disulfide bonds link the two chains together at specific locations [25]. Trypsin cleaves insulin at the following locations B29-Lys and B22-Arg, while chymotrypsin attacks insulin at locations A11-Cys, A14-Tyr, A19-Tyr, B1-Phe, B15-Leu, B16-Tyr, B25-Phe, and B26-Tyr [26] Insulin chains are much more susceptible to the attack by pepsin as the enzyme is capable to cleave the chain at 15 locations (four sites in the region of A13-A19, five spots in A2-A8 segment, and six positions located in the B chain) [26]. Since pepsin action was fast and complete in degrading insulin

held within the EGCG-insulin complex, this could point out to the fact that the hydrogen bound formation and hydrophobic interactions between insulin and EGCG were not sufficient enough to shield the aforementioned vulnerable sites available on insulin chain from the proteolytic action of pepsin, keeping some or all of these sites exposed to the damaging effect of the enzyme. On the other hand, the protective effect of EGCG on insulin seen with SIF was perhaps related in part to the EGCG-insulin complex's three-dimensional structure formation in protecting to some degree the 10 susceptible sites from the degradative action of trypsin (two locations) and chymotrypsin (eight locations). Analysis of data indicated that EGCG-insulin complex could resist the proteolytic degradation imposed by trypsin/chymotrypsin for a period of 7 hours with a concentration of insulin in solution of 20 IU/mL and stored at room temperature (Figure 3). However, a change in temperature from 25°C to 37°C would cause almost a total destruction of insulin, present in the solution in the form of EGCG-insulin complex, by trypsin/chymotrypsin (predicted insulin remaining = 30.56%; 95% CI = -8.62% to 69.75%) (Figure 4).

Thus, EGCG-insulin complex's degradation by trypsin/

chymotrypsin appears to be highly sensitive to a rise in temperature. One explanation to that might be that the vulnerable sites on insulin chain that were normally susceptible to trypsin/chymotrypsin action and were originally shielded by EGCG-insulin complex formation became exposed to the enzymes at higher temperatures (37°C). Perhaps this was related to hydrogen bounds weakening as the temperature increased from 25°C to 37°C [27]. Further investigations are needed to ascertain this temperature-dependent sensitivity of the complex to its denaturation by the enzymes. The implication of this study is that although insulin could form a complex with EGCG at room temperature, shielding its susceptible sites from trypsin/chymotrypsin proteolytic action, this protection lessened greatly and perhaps totally disappeared, at 37°C. Thus, the oral administration of EGCG-insulin complex in a form of enteric coated delivery system (to protect insulin from the action of pepsin) would not be expected to render any improvement in insulin bioavailability over that seen with insulin alone. Furthermore, any freed EGCG from the complex would not be expected to have a significant inhibitory activity on the enzymes (i.e., trypsin and chymotrypsin) *in vivo* because of the presence of salivary proline-rich proteins would protect the digestive enzymes from EGCG inhibitory effect [18]. From all practical points of view, EGCG cannot be expected to protect insulin administered orally from the degradative proteolytic action presents in the GI track.

Conclusions

In summary, the complex formation between EGCG and insulin did not protect insulin from the proteolytic action of pepsin, while EGCG-insulin complex rendered the degradative activity of trypsin and chymotrypsin on insulin less pronounced at room temperature. The stability of insulin in solution as EGCG-insulin complex in the presence of trypsin/chymotrypsin was found to be temperature-dependent. EGCG did not protect insulin from the action of proteolytic enzymes at 37°C, the one expected to be encountered *in vivo*.

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