

Synthesis, Purification, and Biological Activity of AIB Substituted Glucagon and GLP-1 Peptide Analogues

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Introduction

Diabetes: Glucagon-Like Peptide-1

Diabetes mellitus is characterized by multiple metabolic abnormalities. This a chronic disease arises primarily from an inadequate insulin effect. The control of glucose through insulin secretion no longer has its normative modulation. Specifically in type 2 diabetic patients, there is no incretin-mediated augmentation of insulin secretion.¹ There is no control regulated by insulin secretion to control or help mediation of glucose in the case after food consumption. Glucagon-like peptide (GLP)-1 is a gut hormone that stimulates insulin secretion, gene expression, and pancreatic β -cell growth. This mammalian hormone has a 30 amino acid sequence and has been clinically studied to have therapeutic effects as an anti-diabetic agent. These therapeutic actions include glucose-dependent insulintropic action, modulation of gastric emptying, and appetite control.^{1,2} GLP-1 is a product of the glucagons gene and shares 50% homology. This hormone is a potent substance in insulin release mediated by metabolism throughout the daily activity.

GLP-1 is coupled with the hormone glucosedependent insulintropic polypeptide (GIP). Together, the two hormones induce the incretin effect. The presence of GLP-1 helps retain the insulintropic effects of type 2 diabetic patients.² In a recent study, GLP-1 has been shown to have greater insulintropic effect as compared to its coupled hormone, GIP. In a worst case scenario of advanced type 2 diabetes, during hyperglycemia, infusion of GIP had little effect on insulin secretion to counteract the hyperglycemic clamp. However, with the usage of GLP-1, the insulin response was restored to help turnover glucose levels.² Therefore, when there is decrease in secretion of GLP-1, there are negative consequences on metabolism due to the insulintropic effects.

A drawback on Glucagon-Like Peptide as a therapeutic agent is its short duration of action.^{1,2} This limits the hormones effects as a anti-diabetic agent. There is a need to enhance the half-life of the hormone within the body so that its insulintropic effects are prolonged and sustained. The objective of this laboratory rotational study correlates with this fact. Synthetic GLP-1 analogs, along with its analogs of its homolog, Glucagon, will be synthesized and studied in hopes of obtaining an analog with prolonged active duration within the cell.

Exendin-4

Like that of GLP-1, a newly synthesized clinical drug, Exendin-4 has been found to exhibit similar anti-diabetic properties. This is a 39 amino acid peptide first isolated from the Gila Monster lizard. This hormone has been indicated to have improved glucose control over GLP-1.³ Exendin-4 has been shown to stimulate secretion of insulin in the presence of elevated blood glucose concentration, and not during periods of hypoglycemia. This hormone is shown to have been more potent due to its longer duration of activity.⁴ Therefore, it has a more prolonged insulintropic effect. This longer half-life may be due to the extra 8 amino acid residues at the C-terminal tail of the Exendin hormone (residues 31-39).^{3,4} At position 31, there is a Tryptophan residue that has the ability to create a Trp-cage by folding the remaining amino acids in the sequence onto itself.^{4,5} This in effect creates a more stable helical formation, thus giving less chances degradation. In essence, through substitutions within the amino acid sequence of Glucagon and GLP-1, the helical formation can be enhanced, therefore stabilizing the structure thus in effect prolonging the duration that the hormone may be active.

AIB Substitution

Synthetic substitution along the natural amino acid sequence of Glucagon and GLP-1 will be done with an amino acid uncommon in human metabolic synthesis. Naturally, this amino acid is contained in antibiotics synthesized by fungi.⁸ The amino acid is to be used because it has been implicated to be a stronger helix inducer in peptide synthesis.^{7,9} The substitution will be done with α -aminoisobutyric acid or alpha-methyl-alanine (AIB, $\text{H}_2\text{N}-\text{C}(\text{CH}_3)_2-\text{COOH}$).⁹ The residue's effect of inducing helicity is not properly deduced, but the fact that there are two methyls attached to the α -carbon may create additional hydrogen bonding, stabilizing a helical structure.

Experimental Objectives

Exendin-4 exhibits similar antidiabetic effects like that of Glucagon-like peptide (GLP-1).r The Exendin-4 (EX4) peptide, as mentioned above was first isolated from the Gila monster. EX4 displays 53% homology with GLP-1 (1-30 amino acid sequence). Therefore, EX4 has been shown to bind to GLP-1 receptors and it displays itself as having full potency at the GLP-1 receptor.² However, Exendin carries an extra 8 residues (31-39) with a Trp at position 31 displaying the ability to form a Trp-Cage. Because of EX4's Trp-cage formation at residues 31-39, its half-life ($t_{1/2}$) within the cell is increased. The Trp-cage of Exendin is believed to have a stabilizing effect of helix formation, thus gaining a longer presence in the cell due to the cell's decreased ability to degrade the substrate.^{4,5} Thus, the question is posed on how to increase the stability of the natural Glucagon and GLP-1 peptides to match the antidiabetic effects of Exendin without the extra amino acid tail (positions 31-39) forming the Trp-cage. Since AIB has been implicated to have the ability to induce helical formation, substitutions along the respective Glucagon and GLP-1 amino acid sequence may be favored to help stabilize the peptides in hopes of gaining a longer presence

within the cell.^{3,4} Therefore, the objective of this laboratory rotational experiment is to synthesize GLP-1 and its homolog Glucagon by Solid Phase Peptide Synthesis (SPPS) with substitutions along their respective amino acid sequences by the unnatural amino acid, aminoisobutyric acid (AIB). After synthesis, purification and final biological activity will be performed to determine comparable EC50 values of analogs to natural peptides in respect to Glucagon and GLP-1 receptors. The following are AIB substitutions along the amino acid sequences of Glucagon and GLP-1 (Table 1). A representative schematic of the natural amino acid sequence of Glucagon and GLP-1 peptides can be observed in Figures 1 and 2. The amino acid positions are numbering accordingly from N- to C-terminal. Amino acids are chosen to be at positions that may contribute to the overall helical structuring of the peptides.

Peptide AIB Substitutions	Position	Natural Residue
Glucagon		
AIB 16,20 Glucagon		16 R
AIB 16,24 Glucagon		20 Q
AIB 20,24 Glucagon		24 Q
AIB 16,20,24 Glucagon		
GLP-1		
AIB 24,30 GLP-1		22 G
AIB 24,27,30 GLP-1		24 A
AIB 22,24,30 GLP-1		25 A
AIB 22,25,30 GLP-1		26 K
AIB 22,26,30 GLP-1		27 E
AIB 22,24,27,30 GLP-1		30 A
AIB 22,25,27,30 GLP-1		

Table 1: AIB substitutions as indicated in the peptide. The natural amino acids at positions to be substituted by AIB.

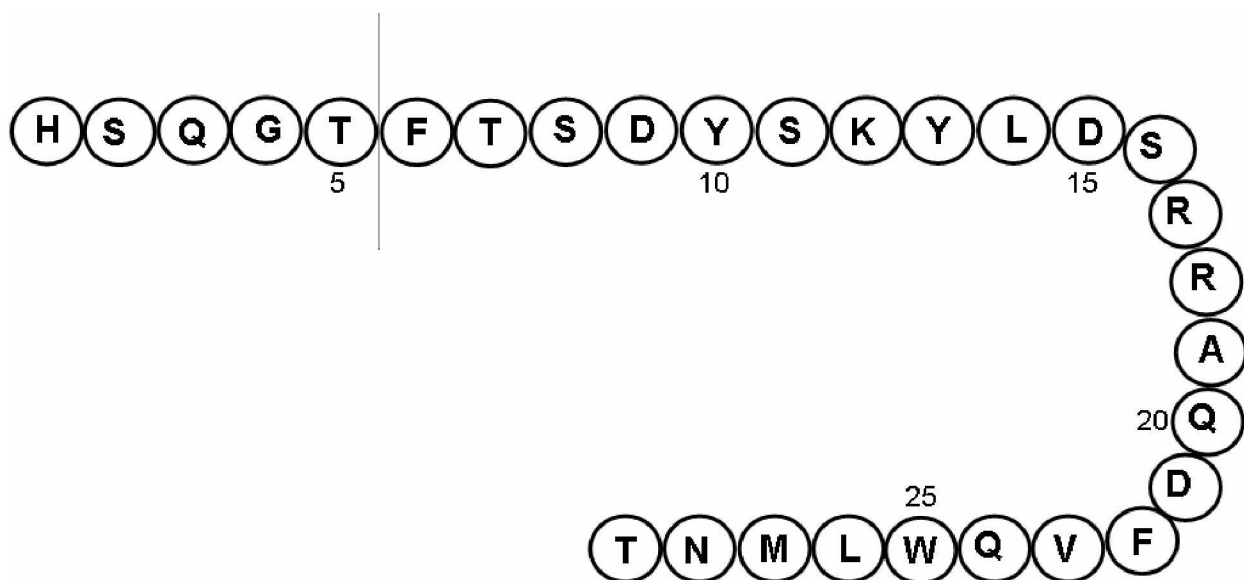


Figure 1: Glucagon peptide amino acid sequence. AIB substitutions will be made at certain positions as described in Table 1.²²

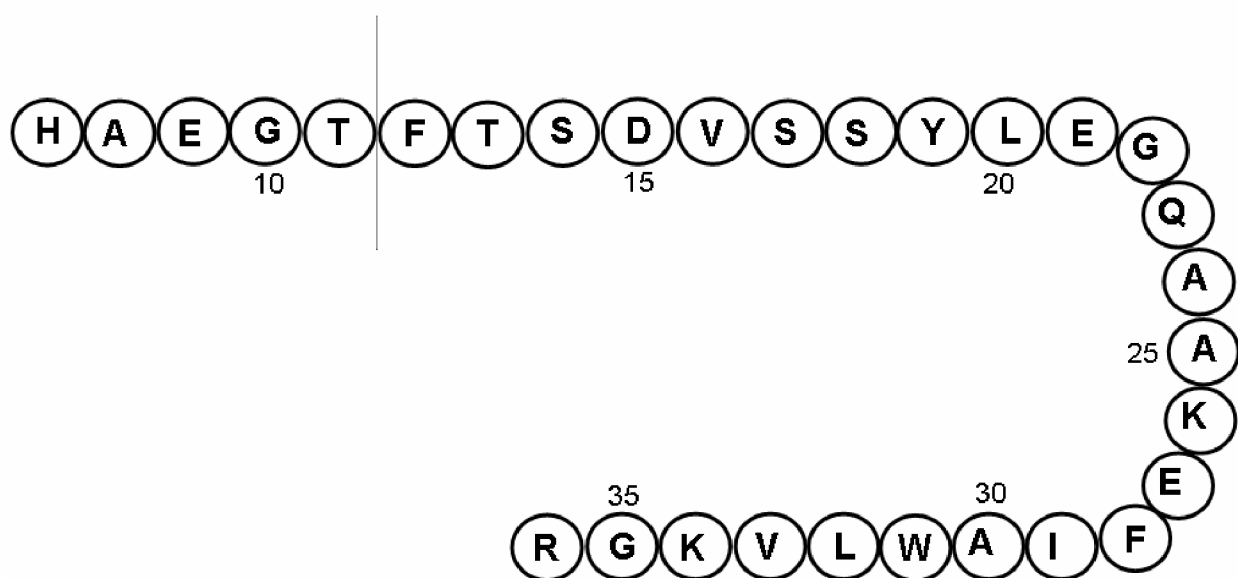


Figure 2: GLP-1 peptide amino acid sequence. AIB substitutions will be made at certain positions as described in Table 1.²²

Methods

Synthesis of the AIB substituted Glucagon and GLP-1 analogs require a number of experimental steps from the starting material of individual amino acids, to final, active, purified sequence of peptide. The sequential chemical procedure found in Solid-Phase Peptide Synthesis (SPPS) was first pioneered by Bruce Merrifield.^{10,12} Coupling individual amino acids in a

sequential in an automated fashion is employed to synthesize natural peptides and various analogs of those natural peptides.¹⁰ The method at present is highly automated, although manual peptide synthesis can still be performed. This method of synthesizing is employed not only because it is automated, but mostly due to the reason that it cuts the time of each individual amino acid coupling down to 20 minutes as compared to hours. The two synthesizers employed in the synthesis of the analogs were the 430A and Biosphere.

Once the peptides are sequenced, a preparatory mass spectroscopic reading of the sample is taken to see whether the intended peptide was correctly synthesized. As most peptides being synthesized, there are incorrect couplings of the amino acids within the sequence. The target product with the correct molecular mass will be interspersed with incorrect product. The differing trace products of peptides from that of the target molecular weight consequently leads to a need to have the correct sequence separated from incorrectly coupled peptides. This is done through High Pressure Liquid Chromatography (HPLC). With proper mobile and stationary phases, chemical separations can occur by differing migration patterns of various peptides.

Once the fraction containing the target peptide is obtained, a biological activity assay is performed using the luciferase assay technique. This assay compares the activity of the analog AIB substituted Glucagon and GLP-1 peptides to that of natural Glucagon and GLP-1.

Solid-Phase Peptide Synthesis

The standard in synthetic peptide manufacturing is attributed to Solid-phase peptide synthesis (SPPS) which allows synthesis of both natural and unnatural peptides. The technique allows the incorporation of the unnatural amino acid, AIB. SPPS proceeds from the C-terminal end of the peptide sequence and ending at the N-terminal.^{10,12} This is unlike cellular protein

synthesis through ribosomal mediation where the production proceeds normally from the N- to C-terminus. There are two major forms of SPPS, Fmoc and t-BOC (tert-ButylOxyCarbonyl). However, in this project, the t-BOC method was performed, thus will be further discussed.¹⁰

In the t-BOC system, the N-termini of the amino acid monomers are protected by the BOC group. This protecting group introduced by chemical modification of the amine functional group to ensure chemoselectivity in a subsequent chemical reaction. This inactivates the N-terminus of the amino acid until it is ready to be coupled in the sequence for the peptide.¹⁰ To ensure high yield of final synthesized peptide product, each amino acid in the sequence is sequentially added in great excess. Coupling of amino acids in a sequential manner is optimized by a series of specific, well-characterized agents. These reagents include: di-isopropylethylamine (DIEA) to neutralize the resin beads and activate the coupling reagent; Dimethylformamide (DMF) as the primary solvent for deprotection, coupling, and washing; O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as a coupling reagent.^{10,11,12}

This synthesis starts with the first amino acid at the C-terminus coupled to a resin bead. Subsequent coupling proceeds by removing the BOC protecting group from the amino acid monomer by acidic conditions through usage of trifluoroacetic acid (TFA).^{10,12} The amino acid in the sequence of the target peptide are then added sequential in an automated fashion. The synthesizers used in the laboratory are the 430A and Biosphere. Each follows the same mechanics, yet differs slightly in the reagents used. After all the residues have been coupled, the final step of the process calls for removal of side-chain protecting groups and the peptide from the resin by incubation in anhydrous hydrofluoric acid.^{10,12,14}

Electrospray Ionization Mass Spectroscopy

Electrospray ionization mass spectroscopy allows for direct analysis of large peptide molecules directly from the liquid phase. The peptide in question is dissolved in a polar solvent, in the case of the Glucagon and GLP-1 analogs, the solvent is acetonitrile (CH_3CN).^{15,17} This technique obtains the molecular mass of the synthesized peptide in comparison to the expected mass from the chemical formula of the analog. In the case of peptides, the mass of all the amino acids in the sequence would be the expected target molecular mass.

The peptide sample is dissolved in acetonitrile and this volatile solvent is inserted into the mass spectroscopic apparatus through a capillary where a high voltage (3 kV) source is applied.^{16,17} This is within the ionization source of the mass spectrometer where the high voltage creates a strong electric field. Once the sample peptide exits the tip of the capillary, it is instantaneously immersed into an aerosol of highly charged droplets of acetonitrile. The solvent evaporates by a nitrogen nebulizing gas, leaving only charged peptide ions, free from the droplets. The ionized peptide is analyzed by passing through the sample orifice into an intermediate vacuum chamber and eventually to the analyzer for mass spectroscopy.^{15,17} Differing ionization levels of hydrogen atoms (H^+) are obtained for the sample peptide. The spectroscopic reading gives a plot of intensity versus m/z where m is the molecular mass and z represents the number of charges. The ionization levels, intensity, and m/z ratio correlate to the mass of the peptide sample.

Electrospray ionization mass spectroscopy is performed as a preliminary analysis after peptide synthesis and cleavage of protecting groups to conclude whether the product contains the target peptide compound. However, peptide synthesis inevitably contains contamination of undesired trace products due to improper coupling. Thus, HPLC is performed to separate the pure target peptide from contamination of various compounds. Afterwards, the fractions suspected of containing the target peptide as indicated by peaks through UV detection are further

analyzed through mass spectroscopy. This reading will ensure the fraction contains pure, desired peptide.

High Performance Liquid Chromatography (HPLC)

Creating synthetic peptides through SPPS inevitably includes incorrectly sequenced peptides along with the intended product. Most of these incorrect peptides are exclusion of amino acids from the target product. The coupling of a sterically hindered amino acids to the growing peptide chain is chemically difficult, thus some of the chains are incomplete. This same principle also applies when having a sterically hindered residue at the end of the peptide chain before the next coupling. Some of the sterically hindered amino acid residues include: Isoleucine, and the unnatural residue, AIB. Therefore, HPLC is employed as a means to separate the various synthesized compounds.

With the proper mobile and stationary phases conditions, HPLC techniques can adequately separate, identify, purify, and quantify chemical compounds. Chemical separations can be accomplished using HPLC by utilizing the fact that the differing peptides have characteristic migration rates in a particular column and mobile phase due to amino acid sequence.¹⁸ This technique specifically refers to preparative HPLC where the main focus is isolation and purification of a target peptide compound from various contaminants. Depending on the relation of target peptide and contaminants, certain chromatographic conditions will elute characteristic peaks of differing compounds within the same solution. The migration through sequential gradient elution of the target peptide and contaminants need to be different enough so the collected elution fraction contains only pure target peptide.¹⁸

The specific HPLC technique utilized for peptide purification is reversed-phased chromatography (RPC). Commonly used mobile phase for RPC of peptides is a gradient of 0.1%

trifluoroacetic acid (TFA) in water (buffer A) to 0.1% TFA in an organic solvent, such as acetonitrile (CH₃CN) (buffer B).¹⁸ The organic solvent solubilizes the peptide, allows detection at approximately 230-240 nm, and can evaporate away from the sample through lyophilization. This process utilizes an analytical column as the stationary phase. As the gradient mobile phase flows through the stationary column, differing components migrate according to non-covalent interactions along the column.¹⁸ As the elution proceeds through the continuously increasing gradient (ratio of buffer A decreases as compared to B), separation of compounds by degree of migration occurs, and the fractions are collected, consequently obtaining pure target peptide compound.

The specific chromatographic conditions in HPLC compound separation is in Table 2:

HPLC Chromatographic Conditions	
Column:	Vydae Cy 5 cm x 25 cm, C4
Gradient Condition:	Buffer A: 0.1% TFA in 10% CH ₃ CN solution in H ₂ O Buffer B: 0.1% TFA in CH ₃ CN 0% to 100% B in 120 minutes Flow Rate: 40 ml/min
Chart Speed:	5 mm/min
UV Detector:	230 nm / 2.0 AUFS
Fraction Size:	0.5 min fractions

Table 2: HPLC chromatographic conditions

If the contamination of undesired compounds seemed to be in abundance, conditions needed to be altered to allow more precise separation. The alterations were the use of a different column and gradient condition (all other conditions remained the same) (Table 3).

HPLC Chromatographic Conditions	
Column:	Vydae Cy 1 cm x 25 cm, C1
Gradient Condition:	Buffer A: 0.1% TFA in 10% CH ₃ CN solution in H ₂ O Buffer B: 0.1% TFA in CH ₃ CN 0% to 100% B in 150 minutes Flow Rate: 40 ml/min
Chart Speed:	5 mm/min
UV Detector:	230 nm / 2.0 AUFS
Fraction Size:	0.5 min fractions

Table 3: Altered HPLC chromatographic conditions.

The change in column and the usage of a longer gradient time of 150 minutes will consequently create a greater separation scheme and prolong the elution gradient. This ensures the target compound is separated sufficiently from contaminants.

Biological Activity, Luciferase Assay

To test for biological activity of the synthesized peptide analogs, the technique of luciferase assay is performed. This type of assay employs the concept of genetic reporters to study peptide expression on a receptor to illicit a signal transduction.^{19,20} The luciferase protein is obtained from firefly beetles which catalyze the luciferin oxidation generating light in its reaction. This technique is used mainly because reporter activity is available immediately upon translation, the assay is rapid, and sensitivity to the light produced is proficient since chemiluminescent reaction of luciferase and luciferin is the only source within the host cell therefore detection is complete without disturbance.²⁰

The AIB substituted Glucagon and GLP-1 analogues are assayed with their natural receptors along with respective wildtype peptides. The biological activity of the analog peptide was compared to wildtype. Analogs having proficient, comparable activity to natural peptide were then further studied to elucidate further biological activity to see whether the substitution at with AIB amino acid truly produced a comparable analog.

The biological activity luciferase assay was performed by other members of the laboratory group. These members were Brian Finan and Dr. Vasily Gelfanov. Since glucagons and GLP-1 are homologs of one another, biological activity assays of the peptides, whether Glucagon or GLP-1 analogs, were done in the presence of both Glucagon and GLP-1 receptors. Assays of transfected cells were performed as well as in mediation with the presence of cAMP. The assay will measure and compare the EC50 value of these analogs. EC50 is the molar concentration of an agonist, which produces 50% of the maximum possible response for that agonist.

Results

The results show a summarized compilation of biological activity assays of all synthesized Glucagon and GLP-1 analogs done during the rotational project. Representative analysis and data of sequential steps in the process of peptide synthesis, mass spectroscopic detection, HPLC purification, and biological assays of two AIB substituted Glucagon analogs are given. The analogs are: di-substituted AIB-20,24-Glucagon and tri-substituted AIB-16,20,24-Glucagon.

Peptide	Glucagon Receptor			GLP-1 Receptor			Fold Difference	
	Average EC50	STDev	n	Average EC50	STDe v	n	Glucago Receptor	GLP-1 Receptor
Glucagon	0.14	0.06	14.00	13.70	4.26	10.00	1.00	1.00
AIB 16,20 Glucagon	0.26	0.06	2.00	13.62	8.92	2.00	2.00	1.00
AIB 16,24 Glucagon	0.18	0.01	2.00	31.81		1.00	1.00	2.00
AIB 20,24 Glucagon	0.34	0.15	2.00	10.59	1.95	2.00	2.00	1.00
AIB 16,20,24 Glucagon	0.42	0.18	2.00	12.15	3.77	2.00	3.00	1.00
GLP-1	2878.03	2510.39	7.00	0.05	0.02	15.00	20557.00	0.00
AIB 24,30 GLP-1	540.28	190.16	2.00	0.26	0.09	2.00	3859.00	0.00
AIB 24,27,30 GLP-1	330.70	208.44	2.00	1.40	0.05	2.00	2362.00	0.00
AIB 22,24,30 GLP-1	1582.89	256.20	2.00	0.11	0.04	2.00	11306.00	0.00
AIB 22,25,30 GLP-1	1315.28	140.21	2.00	0.18	0.05	3.00	9396.00	0.00
AIB 22,26,30 GLP-1	3943.04	1582.00	2.00	0.15	0.04	3.00	28165.00	0.00
AIB 22,24,27,30 GLP-1	174.08	7.61	2.00	0.80	0.41	2.00	1243.00	0.00
AIB 22,25,27,30 GLP-1	2190.37	205.53	2.00	0.80	0.48	2.00	16645.00	0.00

Table 4: Compilation of average EC50 values of various Glucagon and GLP-1 analogs in Glucagon and GLP-1 receptors with comparison to wildtype. AIB substituted analogs are represented as AIB-position of amino acid in sequence-Glucagon (GLP-1).

As stated previously, the EC50 measures the molar concentration of an agonist producing 50% of the maximum possible response for that agonist. For Glucagon, results show that the Glucagon analog containing the AIB substitution at 16 and 24 has comparable EC50 (0.18 M) values to the natural Glucagon peptide (EC50 0.14 M) when in the presence of Glucagon receptor. The next Glucagon analog having a significantly comparable EC50 is AIB 16,20 Glucagon (0.26 M). The analog having the largest difference, thus not a potent analog for the

Glucagon receptor was the tri-substituted, AIB 16,20,24 Glucagon (EC50 0.42 M). These results imply that in the substitution of AIB for natural amino acid residues of Glucagon at positions 16 and 24 will readily accept the replacement, construct the natural helical structure to perform almost as potently as the natural Glucagon peptide (Table 4).

As for GLP-1 peptide (EC50 0.05 M), the analog having significantly comparable EC50 to the natural peptide value is AIB 22,24,30 GLP-1 (0.11 M) with the next closest being AIB 22,26,30 GLP-1 (0.15 M). The analog having the greatest difference in potency is AIB 24,27,30 GLP-1 with a high EC50 value of 1.50 M. It seems that the GLP-1 homolog readily accepts tri-substitutions, however in order for the peptide to function close to natural potency, there are positions more favored over others. This seems to be the residue stretch starting with position 22, 24 or 26, and ending with 30 (Table 4).

ESI Mass Spectroscopy

The following is a representative data set of ESI mass spectroscopic readings. For each synthesized peptide, a preliminary ESI reading was performed to confer whether the product was the correct target peptide by comparison to the expected molecular mass. The molecular mass was obtained by looking at the amino acid sequence. Once the preliminary ESI reading was performed, if the majority of the synthesized product was confirmed to contain the correct target peptide, the product was assumed pure enough to be tested for biological activity. However, as in many of the synthesized peptide analogs, if trace products were detected by having smaller or larger molecular masses, HPLC had to be performed in order to separate out the pure target peptide from the contaminants within the mixture of synthesized products. HPLC is performed and as peaks are detected through the UV source at 230nm, representative elution fractions

occurring at significant peaks were collected in hopes that one of the fractions contains the majority of the correct target peptide and the contaminants were separated out. These fractions are put through a secondary ESI reading in hopes of obtaining a fraction containing the pure target peptide with minimal contaminants. If the fraction is confirmed to contain pure target peptide with only minimal traces of contaminants, the neighboring fractions contained in the peak are assumed to contain target peptide as well and are collected for lypholyzation and assayed for biological activity.

The representative data set is for two synthesized Glucagon analogs: the di-substituted, AIB 20,24 Glucagon (Figure 1,2) and tri-substituted, AIB 16,20,24 Glucagon. Following are their amino acid sequence with expected molecular weights (Table 5).

Peptide	Amino Acid Sequence
Original	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
AIB 20,24 Glucagon	HSQGTFTSDYSKYLDSRRAJDFVJWLMNT
Elemental Composition	C ₁₅₁ H ₂₂₄ N ₄₁ O ₄₇ S ₁
Molecular Mass (amu)	3396
AIB 16,20,24 Glucagon	HSQGTFTSDYSKYLDJRRAJDFVJWLMNT
Elemental Composition	C ₁₅₂ H ₂₂₆ N ₄₁ O ₄₆ S ₁
Molecular Mass (amu)	3395

Table 5: Amino acid sequence of original Glucagon along with di and tri AIB substituted. The J in the sequence represents the position in which the original residue is replaced by AIB. The elemental composition of the sequence is also given as well as the expected molecular mass.

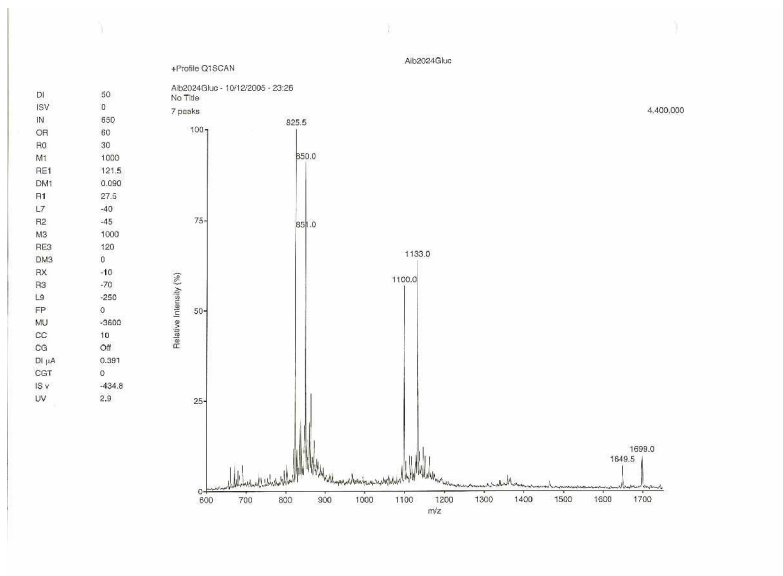


Figure 1: Preliminary ESI reading of synthesized AIB 20,24 Glucagon product. The readings give different H^+ ionization levels. The expected molecular mass should fall at the representative ionization levels: 850.0 m/z (+3), 1133.0 m/z (+4), and 1699.0 m/z (+5). These peaks represent the correct molecular mass of 3396 amu. However, there are noticeable contaminations represented by other peaks occurring at: 825.5, 851.0, 1100.0 and 1649.5 m/z.

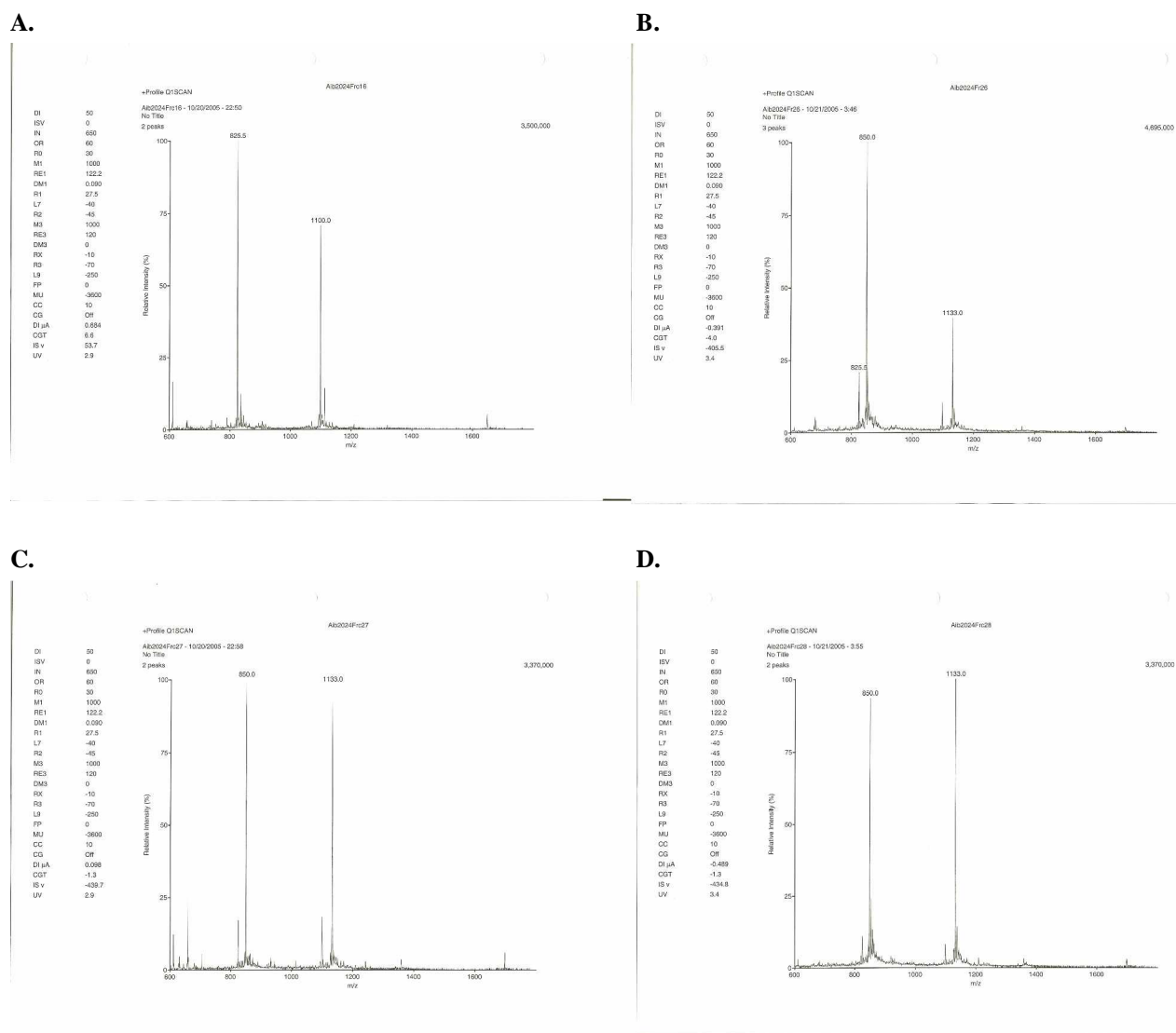


Figure 2: Secondary ESI readings of eluted fractions after HPLC column separation in order to obtain pure target analog, AIB 20,24 Glucagon. **A.** Fraction 16 ESI reading. Peaks are observed at 825.5 and 1100.0 m/z. However, these are peaks representative of a contaminant product, not the target product. **B.** Fraction 26 ESI reading. Correct peaks for the target peptide are observed at 850.0 and 1133.0 m/z. However, there are still peaks of trace contaminants at 825.5 and 1100.0 m/z. **C.** Fraction 27 ESI reading. Again, peaks for the target peptide are observed at 850.0 and 1133.0 m/z at higher intensities implying that most of the peptide in solution is the correct Glucagon analog. However, there are still peaks of trace contaminants as seen in fraction 26. **D.** Fraction 28 ESI reading. Peaks for the target peptide are observed at 850.0 and 1133.0 m/z at high intensity. Trace contaminants, like that in fraction 26 and 27 are still observed, but are minimal.

After the secondary ESI reading, the fractions deemed to contain significantly pure peptide were fractions: 26, 27, and 28 (Figure 4B,C,D). Fraction 16 only contained a contaminant

product having the molecular mass of 3298 amu which was not the expected target mass of 3396 amu (Figure 4A). This trace product could have occurred as a result of improper amino acid coupling. There seemed to be a deletion, since the difference in mass is consistent to the mass of a single amino acid. This deletion could have happened due to steric hinderance of either the amino acid on the elongated peptide chain or the residue to be couple. In either case, the steric hinderance provided by the side chains prevented correct coupling attributing to lower yield of the target peptide. Fractions 26, 27, and 28 retained noticeable contaminant product by peaks representing incorrect masses at 3298, 3663, and 3950 amus attributing either for a deletion or addition of an amino acid. However, these contaminants were of low intensities, therefore minimal trace amounts. This deemed that the fractions sufficiently contained the majority target peptide. Fractions 26, 27, and 28 were pooled to be lypholyzed and furthermore assayed for biological activity of AIB 20,24 Glucagon.

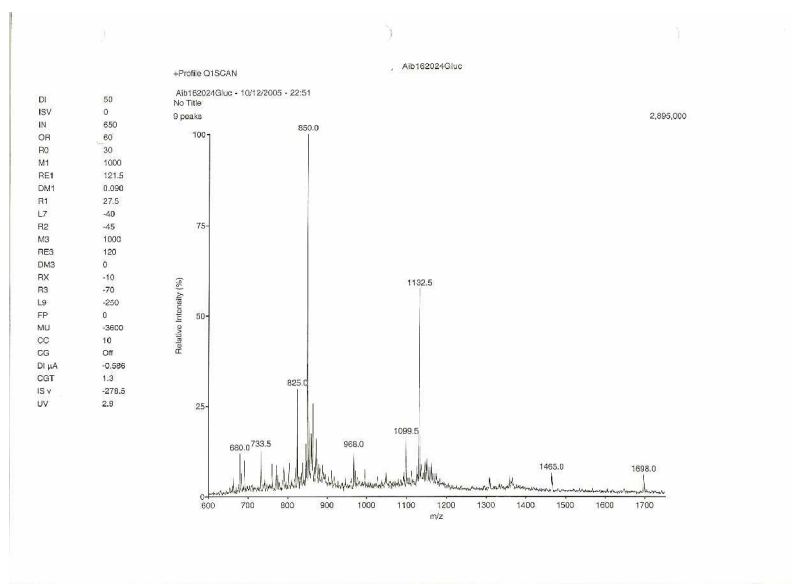


Figure 3: Preliminary ESI reading of synthesized tri-substituted AIB 16,20,24 Glucagon product. The readings give different H^+ ionization levels of the peptide or possible peptides in question. The expected molecular mass should fall at the representative ionization levels: 680.0 m/z (+2) 850.0 m/z (+3), 1132.5 m/z (+4), and 1698.0 m/z (+5). These peaks represent the correct molecular mass of the target peptide at 3395 amu. However, there are

noticeable trace contaminations represented by other peaks occurring at: 733.5, 825.0, 968.0, 1099.5, and 1465.0 m/z. These represent contaminant molecular masses.

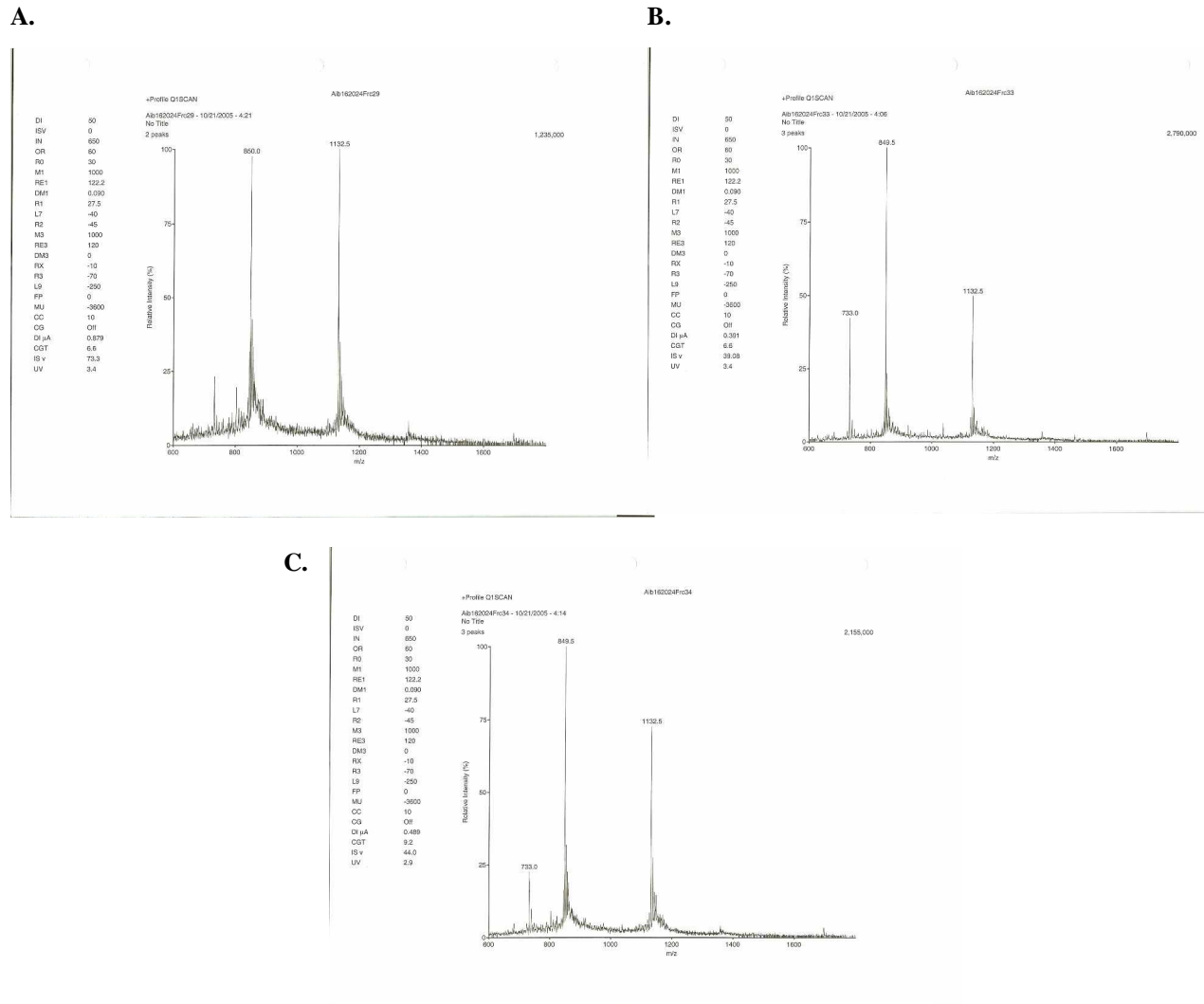


Figure 4: Secondary ESI readings of eluted fractions after HPLC column separation in order to obtain pure target analog, AIB 16,20,24 Glucagon. **A.** Fraction 29 ESI reading. Correct peaks are observed 850.0 and 1132.5 m/z. However, there is an abundance of contamination along the spectrum. Multiple peaks are observed at fairly significant intensities. Peaks are observed at 733, 800 and 1350 m/z (not labeled). The peaks represent contaminant products. **B.** Fraction 33 ESI reading. Correct peaks for the target peptide are observed at 850.0 and 1132.5 m/z with significant intensities. However, there are still peaks of a trace contaminant at 733.0 m/z. This peak represents a contaminant product not well separated from the target peptide. **C.** Fraction 34 ESI reading. Again, peaks for the target peptide are observed at 850.0 and 1132.5 m/z, however at lower intensities. However, there are still peaks of a trace contaminant seen in fraction 33 at 733.0 m/z. However, its intensity is much lower.

Fractions 33 and 34 were deemed to contain the majority of the target peptide. There was a peak representing a contaminant at 733.0 m/z, with a molecular mass of 3660 amu (Figure 4B,C). This can be attributed to an addition of a residue in the synthesis process. However, these peaks were of lower intensities, thus it would make insignificant difference for detection during biological activity assays. Fraction 29 was not used because the presence and intensities of contaminants were much higher than that of fractions 33 and 34 (Figure 4A). Along with fraction 33 and 34, fraction 32 of the HPLC elution was also collected and pooled with the previous two. Fraction 32 was contained within the peak in the HPLC UV detection source, therefore, assumed to also contain the target peptide. The three fractions are pooled and lyophilized to be tested for biological activity of AIB 16,20,24 Glucagon.

The rest of the peptides synthesized were analyzed through ESI mass spectroscopy in the same manner. There was a preliminary then secondary ESI reading after HPLC purification. This process was to ensure that the target peptide sample being sent to test for biological activity by luciferase assay was pure to a high enough degree.

Luciferase Biological Activity Assay:

Below are luciferase assay tests of the synthesized and purified Glucagon and GLP-1 analogs. These tests were performed by Brian Finan and Dr. Vasily Gelfanov. The logarithmic graphs represent genetic reporter gene luciferase as it produces a chemiluminescent reaction in the presence of its substrate luciferin while coupled to the analog and its respective receptor. The analog peptides as tested in the presence of both Glucagon and GLP-1 receptors since the two substrates are known homologs.

Figure 5 contains the biological activity test results of the previous Glucagon analogs, AIB 20,24 Glucagon and AIB 16,20,24 Glucagon. The plot gives the peptide concentration compared to counts per second (cps) of the luciferase/luciferin chemiluminescent reaction as observed in the light detector. This reaction represents the activity and potency measured in EC50 of the analogs compared to the natural substrate in receptor signaling.

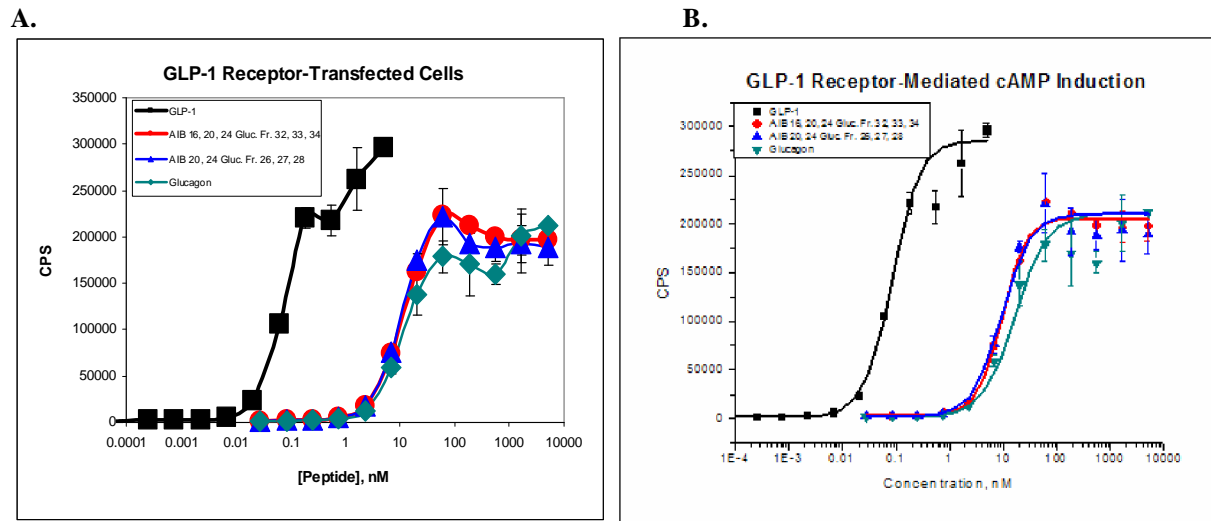


Figure 5: Biological activity test results of Glucagon analogs, AIB 20,24 Glucagon and AIB 16,20,24 Glucagon compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of GLP-1 receptor-transfected cells. **B.** Activity in the presence of GLP-1 receptor-mediated by cAMP induction.

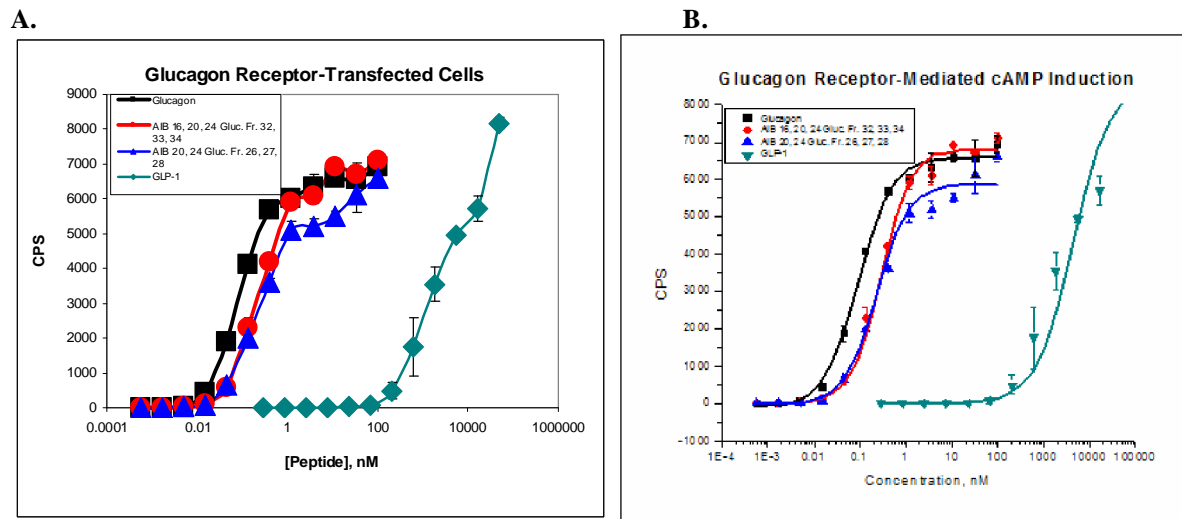
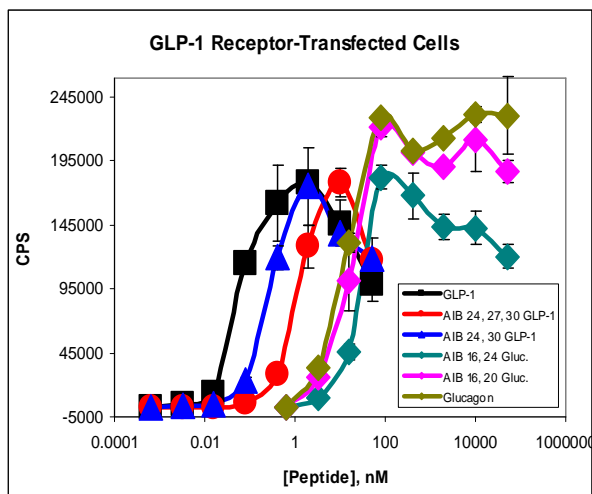


Figure 6: Biological activity test results of Glucagon analogs, AIB 20,24 Glucagon and AIB 16,20,24 Glucagon compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of Glucagon receptor-transfected cells. **B.** Activity in the presence of Glucagon receptor-mediated by cAMP induction.

As represented in the graphs, the Glucagon analogs are not responsive in the GLP-1 receptor. The activity is not comparable with natural GLP-1 peptide. However, in the Glucagon receptor, the analogs show the same characteristic activity curve as that of the natural Glucagon peptide when peptide concentrations are increased. Although the activity curve is not exactly the same, they are similar, thus implying possibility of having comparable EC50 values. However, when looking at further analysis of the analogs being potent in the glucagons receptor, the di-substituted analog, AIB 16,24 Glucagon showed the greatest similarity with comparable EC50 value of 0.18 M to that of 0.14 M of natural Glucagon peptide (Table 3).

A.



B.

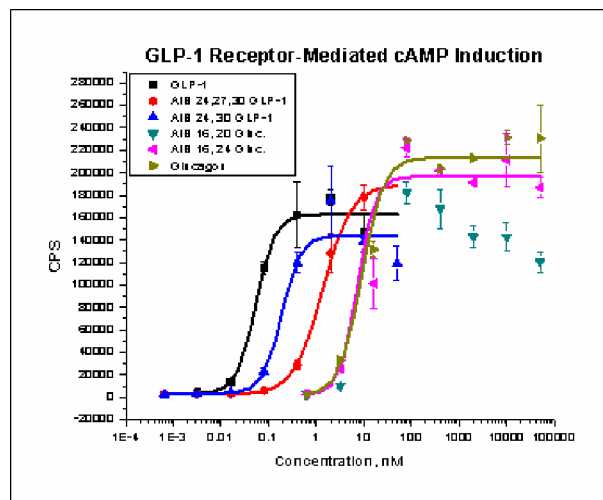


Figure 7: Biological activity test results of Glucagon analogs, AIB 16,20 Glucagon and AIB 16,24 Glucagon as well as GLP-1 analogs, AIB24,27,30 GLP-1 and AIB24,30 GLP-1. These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of GLP-1 receptor-transfected cells. **B.** Activity in the presence of GLP-1 receptor-mediated by cAMP induction.

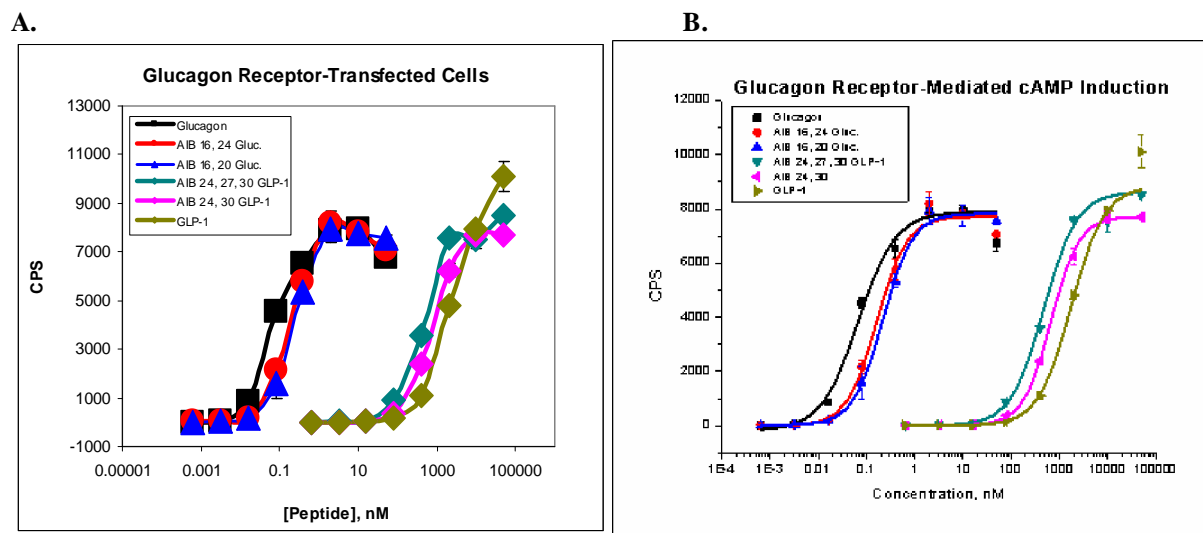


Figure 8: Biological activity test results of Glucagon analogs, AIB 16,20 Glucagon and AIB 16,24 Glucagon as well as GLP-1 analogs, AIB24,27,30 GLP-1 and AIB24,30 GLP-1. These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of Glucagon receptor-transfected cells. **B.** Activity in the presence of Glucagon receptor-mediated by cAMP induction.

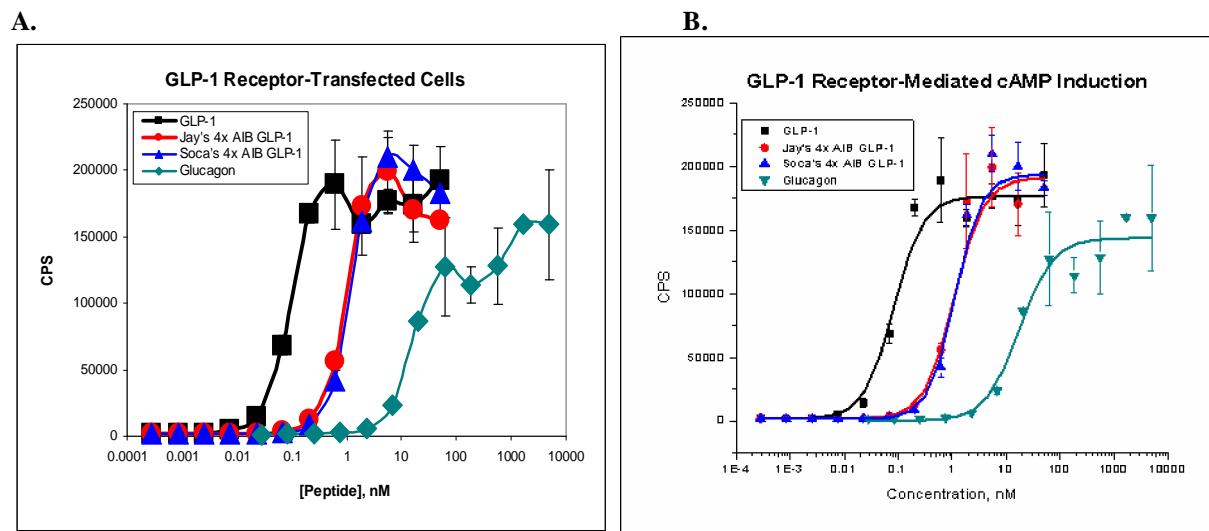
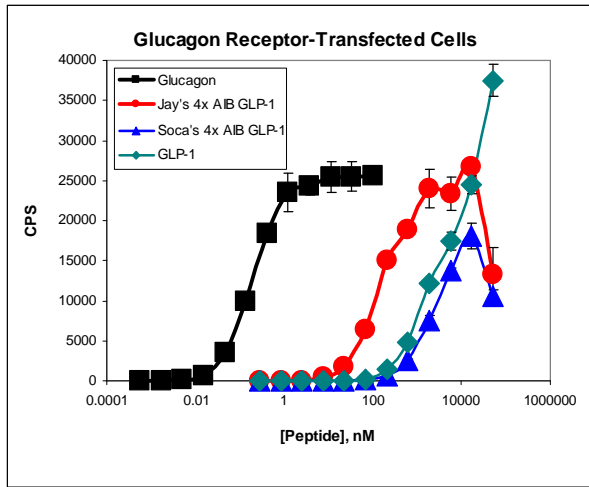


Figure 9: Biological activity test results of 4-AIB-substituted GLP-1 analogs, Jay's 4x AIB GLP-1 (AIB 22,24,27,30 GLP-1) and Soca's 4x AIB GLP-1 (AIB 22,25,27,30 GLP-1). These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of GLP-1 receptor-transfected cells. **B.** Activity in the presence of GLP-1 receptor-mediated by cAMP induction.

A.



B.

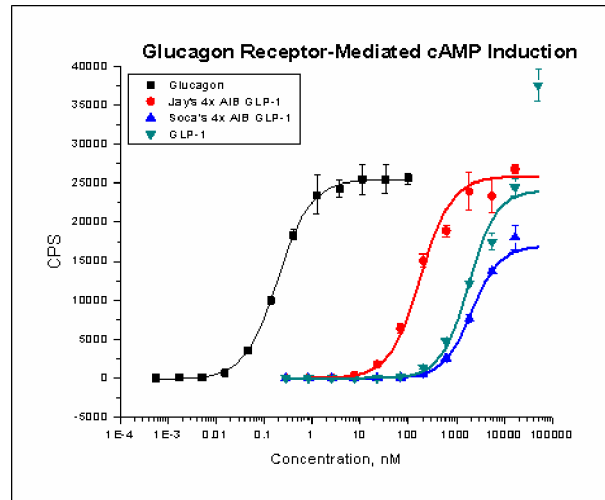
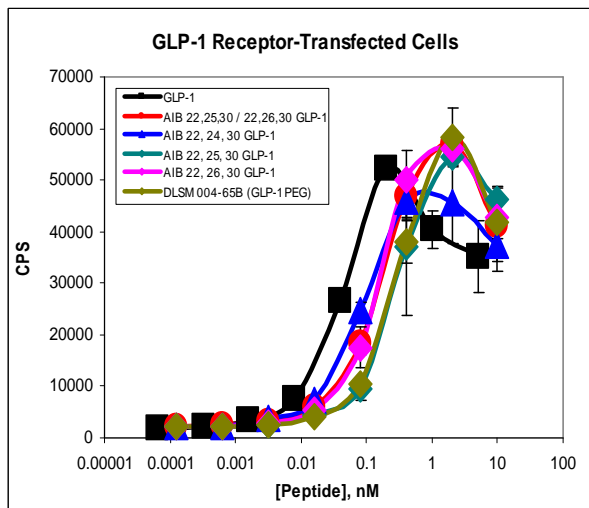


Figure 10: Biological activity test results of 4-AIB-substituted GLP-1 analogs, Jay's 4x AIB GLP-1 (AIB 22,24,27,30 GLP-1) and Soca's 4x AIB GLP-1 (AIB 22,25,27,30 GLP-1). These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of Glucagon receptor-transfected cells. **B.** Activity in the presence of Glucagon receptor-mediated by cAMP induction.

A.



B.

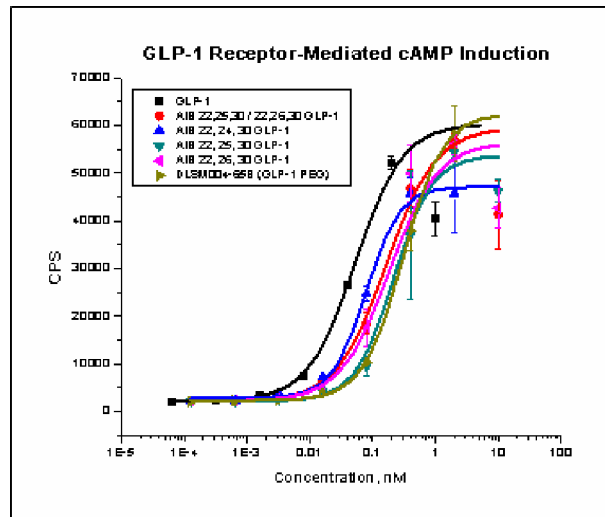


Figure 11: Biological activity test results of multiple tri-AIB-substituted GLP-1 analogs: AIB 22,24,30 GLP-1, AIB 22,25,30 GLP-1, AIB 22,25,30 GLP-1, and an accidental mixture of peptides, AIB 22,25,30 GLP-1/AIB 22,25,30 GLP-1. These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of GLP-1 receptor-transfected cells. **B.** Activity in the presence of GLP-1 receptor-mediated by cAMP induction.

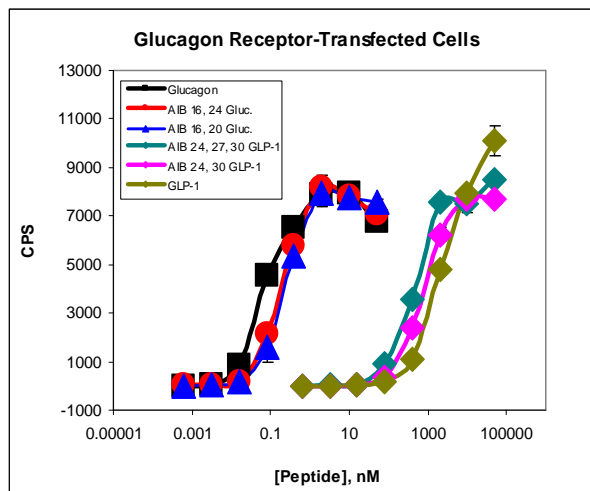
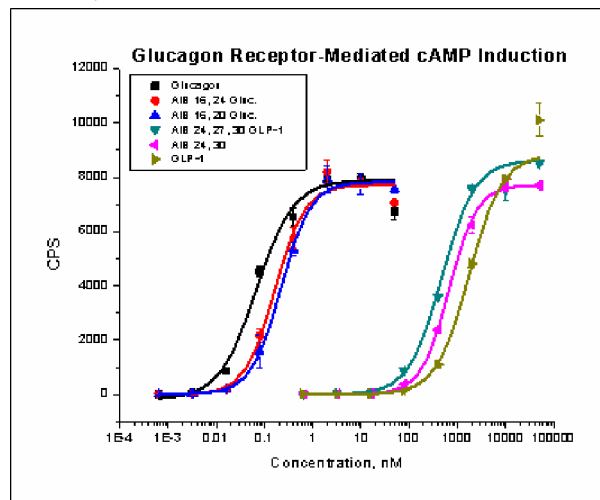
A.**B.**

Figure 12: Biological activity test results of multiple tri-AIB-substituted GLP-1 analogs: AIB 22,24,30 GLP-1, AIB 22,25,30 GLP-1, AIB 22,25,30 GLP-1, and an accidental mixture of peptides, AIB 22,25,30 GLP-1/AIB 22,25,30 GLP-1. These were compared to natural Glucagon and GLP-1 peptide. **A.** Activity in the presence of Glucagon receptor-transfected cells. **B.** Activity in the presence of Glucagon receptor-mediated by cAMP induction.

Discussion:

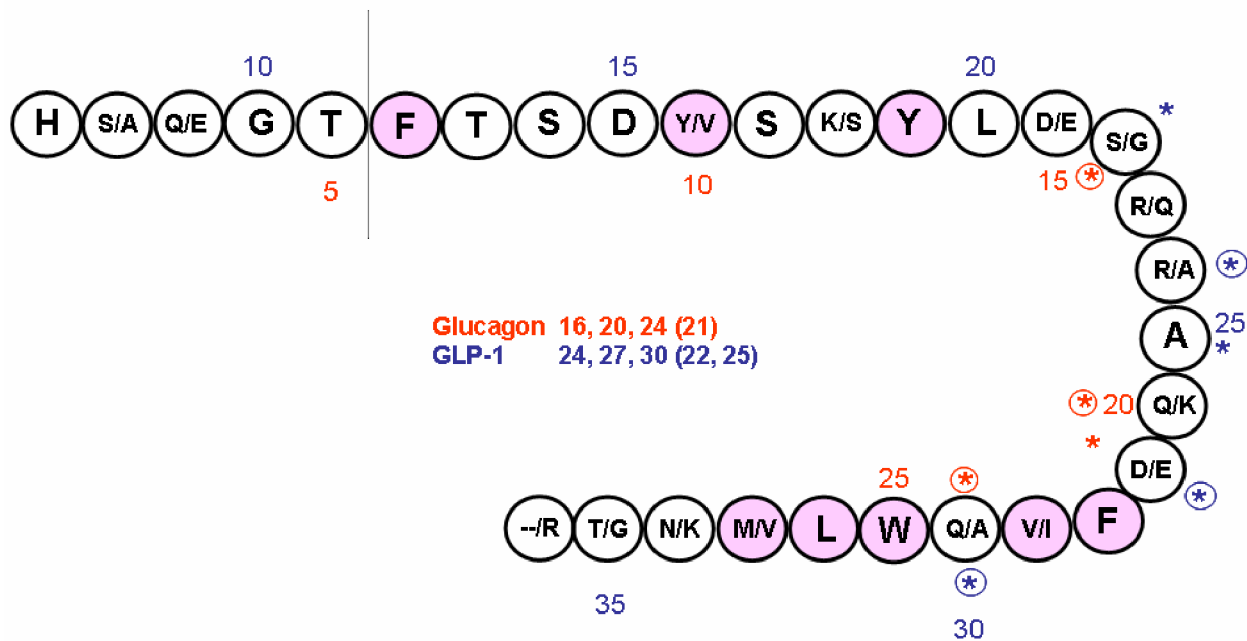


Figure 13: Glucagon & GLP-1 peptide amino acid sequence. Changes by AIB substitutions have been shown by asterisks for AIB 16,20, 24 Glucagon and AIB 24,27,30 GLP-1. ²²

It seems that AIB substitutions for the Glucagon analogs favored di-substitutions as compared to tri-substitutions as seen in GLP-1 analogs when comparing EC50 values. However, when looking at the tri-substituted Glucagon analogs, their EC50 values are not drastically different from that of natural Glucagon peptides. The tri-substituted Glucagon analogs can be said to have comparable significance to that of the tri-substituted GLP-1 analogs.

When looking at the GLP-1 analogs, the tri-substituted peptides exhibit EC50 values significantly comparable to that of the natural peptide. The stretch of amino acid residues in the sequence attributing to favorable AIB substitutions occurs from 22 to 30. At this stretch, tri-substitutions with various combinations were seen at positions 22, 24, 25, 26, 27 and 30. Favorable substitutions giving close comparable EC50 values occurred when the tri-substituted AIB's were in place at certain intervals within the amino acid sequence. The interval of having

either one or two natural amino acid in the sequence before an AIB may have contributed to helical formation and stabilization. The intervals may have given a chance for stabilization that probably would not have occurred if AIB were placed sequentially next to each other. This would have cause steric hinderance and repulsion, thus destabilizing a helix. Further investigation in biological assys and crystallization will shed insight on the exact characteristics of the analogs due to the intrinsic properties of the AIB amino acid

Further Investigation

According to the results of biological Activity through Luciferase assays, several Glucagon and GLP-1 analogs had proficient activity comparable to their respective natural/wild type peptides. Further investigation on these analogs will be performed with collaboration of the Zhang Lab, in the Spring 2006 semester laboratory rotation. Through various crystallization screenings containing differing conditions, the analog peptides will hopefully form a crystal to have their structure analyzed and eventually solved. The Zhang laboratory has the equipment to facilitate crystallization techniques that will foster the possible formation of crystals through crystallization kits such as: Crystal Screen I & II, Wizard I & II, and Index 1-96. From recently and previously synthesized Glucagon and GLP-1 analogs, crystallization studies will be performed on mono-, di-, and tri- AIB substituted analogs.

AIB Substitution	Analog	
	Glucagon	GLP-1
mono-	AIB 16	AIB 22
di-	AIB 16,24	AIB 24,30
tri-	AIB 16,20,24	AIB 22,24,30 AIB 22,26,30

Table 2: Mono-, di-, and tri- AIB substituted Glucagon and GLP-1 analogs studied for crystallization.

Crystallization of these analogs will serve to answer questions of how the peptides naturally fold given proper conditions. The crystals will help to see whether the expected α -helix exists in the structure and how the AIB substitutions help in the formation and stabilization of the helix. Crystallization will be done with 10 mg/ml concentration of peptides dissolved in water in 96 well plates with Crystal Screen I & II, Wizard I & II, and Index 1-96 conditions. A set will be situated at room temperature at 25°C while another in a cold room 4°C.

References:

1. Holst, Jens Juul and Catherine Orskov. The Incretin Approach for Diabetes Treatment. Modulation of Islet Hormone Release by GLP-1 Agonism. *Diabetes*, Supplement 3, December 2004. **53**, S197-S204.
2. Holz, George G. and Oleg G Chepurny. Glucagon-Like Peptide-1 synthetic Analogs: New Therapeutic Agents for Use in the Treatment of Diabetes Mellitus. *Current Medicinal Chemistry*, 2003. **10**, 2471-2483.
3. Hudson, F. Michael and Niels H. Anderson. Exenatide: NMR/CD Evaluation of the Medium Dependence of Conformation and Aggregation State. *Biopolymers (Peptide Science)*, 2004. **76**, 298-308.
4. Neidigh, Jonathan W., R. Matthew Fesinmeyer, Kathryn S. Prickett, and Niels H. Andersen, Exendin-4 and Glucagon-like-peptide-1: NMR Structural Comparisons in the Solution and Micelle-Associated States. *Biochemistry*, 2001. **40**, 13188-13200.
5. Qui, Linlin, et al. Smaller and Faster: The 20-Residue Trp-Cage Protein Folds in 4 us. *J. Am. Chem. Soc.*, 2002. **124**, 12952-12953.
6. Lin, Jasper C., Bipasha Barua, and Niels H. Andersen. The Helical Alanine Controversy: An (Ala)₆ Insertion Dramatically Increases Helicity. *J. Am. Chem. Soc.* 2004, 126, 13679-13684.
7. Heaton, Joanne H.; And Thomas D. Gelehrter. Derepression of Amino Acid Transport by Amino Acid Starvation in Rat Hepatoma Cells. *The J. Of Bio. Chem*, 1977. **252**:9, 2900-2907.
8. Aparna, K.; S. S. Krishnamurthy and M. Nethaji. Amino Acid-Lanthanide Interactions. Crystal Structures Of Lanthanide Complexes Of α -AminoIsobutyric Acid (Aib), [La₂(Aib)₄(H₂O)₈] (ClO₄)₆ and [Pr₂(Aib)₄(H₂O)₈]Cl₆-H₂O. *Polyhedron*, 1994. **13**:21, 2993-2991.
9. Banerjee, Arindam; Animesh Pramanik, Surajit Bhattacharjya, and P. Balaram. Omega Amino Acids in Peptide Design: Incorporation into Helices. *Biopolymers*, 1996. **39**,769-777.
10. Merrifield, R. B. Solid Phase Peptide Synthesis. **I**. The Synthesis of a Tetrapeptide. Rockefeller Institute, New York 21, N.Y. January 31, 1963.
11. Sarin, Virender K.; Stephen B. H. Kent, James P. Tam, And R. B. Merrifield. Quantitative Monitoring of Solid-Phase Peptide Synthesis by the Ninhydrin Reaction. *Analytical Biochemistry*, 1981. **117**, 147-157.

12. Merrifield, R.B. Solid-Phase Peptide Synthesis III. An Improved Synthesis of Bradykinin. *Rockefeller Institute*, New York City. Solid-Phase Synthesis Of Bradykinin, 1964. 3:9.
13. Peptide Synthesis – Chemistry and Modifications. Thermo Electron Corporation, Manual. 2003.
14. Boc Resin Cleavage and Deprotection. Novabiochem, Manual. Calbiochem-Novabiochem AG, 2001.
15. Gilman, Jessica; and Courtney Mashburn. Electrospray Ionization Mass Spectrometry. 17 Sept., 2002 Chemistry 5181. PPT Presentation.
16. Beaver, Melinda; and Erin Wood. Hewlett-Packard 5989 Electrospray Ionization Mass Spectrometer, Manual. 11 Sept., 2003.
17. S. Penco et al. Identification of an import signal for, and the nuclear localization of, human lactoferrin. *Biotechnology Applied Biochemistry*, 2001. 34;151.
18. High Performance Liquid Chromatography (HPLC): A Users Guide. University of Kentucky, Dept. of Pharmacy. <http://www.google.com/search?q=HPLC&start=0&ie=utf-8&oe=utf-8&client=firefox-a&rls=org.mozilla:en-US:official>
19. Ju, Sung-Kyu; Jung-Hyun Park, Shin-Young Na, Kwan-Hee You1, Kil Lyong Kim, and Myung-Kyu Lee. Determination of Rat Leptin Activity *In Vitro* Using a Novel Luciferase Reporter Assay. *Mol. Cells*, 2001. 12:1, 131-136
20. Luciferase Assay System. Instructions For Use Of Products E1483, E1500, E1501, E1531, E4030, E4530 AND E4550. Promega, Technical Bulletin. Manual, 2005.
21. Luciferase Assay Kit. Instruction Manual, Catalog #219020. Revision #113003a.
22. Glucagon and GLP-1 Amino Acid Sequence PPT Slide. Dr. Richard Dimarchi, Dec, 2005.