

**INDIANA UNIVERSITY BLOOMINGTON**  
**CHEMISTRY DEPARTMENT**

**Synthesis of insulin analogues**

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April 24<sup>th</sup>, 2006

## **ABSTRACT**

Diabetes is the disease caused by the presence of a high concentration of glucose in the blood due to a malfunction of the hormone insulin. After the discovery of insulin more than eighty years ago, different kinds of commercial insulin preparations have been developed, however none of them is able to replicate the action of normal insulin, and many have developed side effects in the patients. Insulin analogues that can mimic insulin functions more effectively are under investigation. The chemical synthesis of these analogues is necessary in order to study their function; however the current techniques used give low yields and purity.

In the present work, we propose a new process to produce the interchain disulfide bonds after the chemical synthesis of the A and B chain of insulin. This procedure is expected to occur in higher yields than the reported ones.

## 1. INTRODUCTION

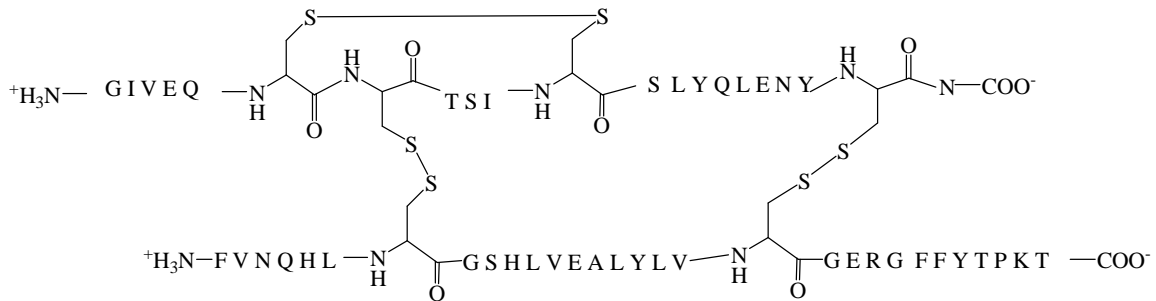
Insulin is a hormone produced in the  $\beta$ -cells of the pancreas. It regulates the uptake of glucose into the cells from the blood to use as fuel, the formation of glycogen for storage in cells, and many anabolic processes like cell growth and cellular protein synthesis<sup>i</sup>. Insulin is produced as a response of a high concentration of glucose that occurs after eating a meal. However, when the level of glucose comes down to the usual physiological value, insulin release from  $\beta$ -cells slows or stops so glucose returns to the normal 'fasting' level.

High levels of glucose in the blood are produced by a malfunction of insulin. This hyperglycemia is the medical disorder known as Diabetes Mellitus. There are two types of Diabetes: Type 1 is caused by a decrease or a complete cease in the production of insulin due to the destruction of  $\beta$ -cells, and Type 2 occurs when the body produces an insufficient amount of insulin or the insulin produced does not function properly<sup>ii</sup>.

### **Structure of Insulin**

Human insulin monomer, which is the biologically active form of insulin, consists of two side chains called A-chain and B-chain. The A-chain has 21 aminoacids whereas the B-chain has 30 aminoacids. These two chains are linked together by disulfide bonds formed between A7-B7 and A20-B19. In addition, the A-chain contains an intra-chain disulfide

bridge between A7 and A11<sup>iii</sup> (**Figure 1**). These disulfide bonds play an important role in the construction of its three dimensional structure.



**Figure 1. Human Insulin**

Insulin can dimerize in the presence of micromolar concentrations of zinc ions, and these dimers can associate into hexamers.

### Treatment of Diabetes

Because the structure of insulin is highly conserved among species, the first sources of insulin were the ones produced by cows or pigs (bovine and porcine insulin differs from human insulin in two and one aminoacid, respectively). However, allergic reactions were developed in patients due to the impurity of the mixture or to the source itself. The employ of genetic engineering techniques that uses microorganisms to biosynthesize proteins, has lead to the production of human insulin (Humulin<sup>®</sup>, Eli Lilly and Company) which does not cause impurity reaction problems. Actually, there are a few patients that still use insulin from animals.

Insulin replacement consists of prandial insulin, basal insulin, and a correction-dose insulin supplement<sup>iv</sup>. Prandial insulin is given to mimic the high levels of insulin produced after the intake of food, basal insulin mimics the small and constant concentration of insulin present within the cells, and correction-dose regulates pre-meal or between-meal hyperglycemia<sup>v</sup>.

Humulin<sup>®</sup> R (regular insulin) had nothing added to change the speed or length of its action. It takes effect rapidly and has a short duration of activity (4 to 12 hours)<sup>vi</sup>. Because of this, it was necessary to develop long-acting preparations to reduce the number of injections necessary to fulfill the insulin requirements. Neutral protamin Hagedorn (NPH, Humulin<sup>®</sup> N) and insulin zinc (Lente, Humulin<sup>®</sup> L) were introduced. These are intermediate-acting insulins with a longer duration of activity (up to 24 hours) than that of regular insulin. These are prandial and basal insulins.

Although these types of insulins improved the life of the patients, most of them have developed hypoglycemia (low levels of glucose in blood) as a consequence of high levels of insulin during the fasting state, and continue to exhibit hyperglycemia, mainly in the postprandial state. As a consequence, new kinds of insulins have been studied. These are called insulin analogues.

### **Insulin analogues**

Insulin analogues are insulins whose structure has been modified in order to improve their absorption, distribution, metabolism and excretion.

The low absorptivity of regular insulin is attributed to the fact that it exists in its hexameric form in the bottles supplied from pharmacy. **Rapid-acting insulin analogues**, which are insulins unable to form hexamers in solution, produce a peak in the activity in the first hour of injection so they increase the insulin concentration in the postprandial state<sup>vii</sup>. Insulin Lispro (Humalog<sup>®</sup>, Eli Lilly and Company) and insulin Aspart (NovoLog<sup>®</sup>, Novo Nordisk) are two rapid-acting insulin analogues.

**Long-acting analogues**, like insulin Glargine (Lantus<sup>®</sup>, Aventis) and insulin Detemir (Levemir<sup>®</sup>, Novo Nordisk), that possess a isoelectric point different than native insulin, are absorbed and released within hours in the body, so they can act as a replacement of basal insulin needs in the fasting state.

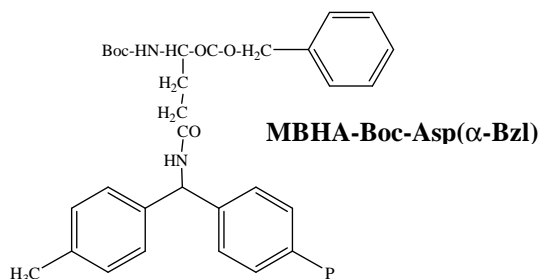
Although great improvements have been made, the production of new analogues that can replicate normal insulin secretion is still under investigation.

## **Production of insulin**

Chemical synthesis of insulin was achieved in the mid-1960s but its high cost makes the production of insulin in a large scale commercially impossible. However, in order to obtain new insulin analogues in the laboratory using a strain of *E. coli*, it is first necessary to study their mode of action. The chemical synthesis of insulin analogues is an important preliminary technique used in the production of these analogues in a small scale.

The most challenged task in the synthesis of insulin is the correct formation of the disulfide bonds after the production of the A and B chains. A few techniques have been developed<sup>viii,ix,x</sup> for the formation of these disulfide bond interchains. In the case of insulin, high yields have not been obtained using these techniques.

The present work proposes a new method for the formation of the disulfide bonds between the chains, following the solid phase synthesis of each chain (**Scheme 1**).



### **Scheme 1. Production of insulin**

The process illustrated in **Scheme 1** consists of five (or four) steps. Step 1 and 2 involve the solid phase synthesis of each chain using the traditional t-Boc methodology on a peptide synthesizer. Step 3 is used to obtain the sulfite group attached to the sulfur in a cysteine side chain when required. Step 4 is the formation of one disulfide interchain bond between A20 and B19. This bond is constructed by reaction of the S-sulfitecysteine at position B19 (peptide **c**) or A20 (peptide **b**) with the thiol group of the cysteine side chain



at position A20 (peptide **a**) or B19 (peptide **d**), respectively. Finally, the formation of the intrachain disulfide bond between A6 and A11 and the second interchain disulfide bond between A7 and B7 (step 4) are expected to occur by reaction with iodine, I<sub>2</sub>, which has been proven to cleave Ac<sub>m</sub> protecting groups<sup>9</sup>. Ac<sub>m</sub> groups were chosen because they are stable against strong acids such as HF<sup>9</sup>, which is used to cleave the peptide from the resin during step 2.

It is necessary to mention, that the A-chain analogues produced contain Aspartate instead of Serine at position 12. This modification has been introduced by Zack Kaur at the DiMarchi laboratory in order to improve the solubility of the chain. The proposed process (**Scheme 1**) will be studied using this modification. When the appropriate set of conditions will be determined to produce insulin, the same conditions will be followed to produce human insulin, and different analogues.

This report show the preliminary steps in the evaluation of the best sets of conditions required to obtain the disulfide bonds in the formation of insulin analogues.

## **2. MATERIALS AND METHODS**

## **2.1. General**

### **Standard solid phase peptide synthesis**

All amino acids were protected with t-Boc and purchased from Midwest Biotech. The cysteine thiol groups at positions A6, A7, A11 and B7 were protected with acid stable AcM groups. The synthesis was carried out on an Applied Biosystem 430A Peptide Synthesizer.

### **HPLC analysis**

Analytical HPLC was carried out using a on a 46 x 5 cm Zorbax C8 column, which was eluted using a linear gradient of 90 % CH<sub>3</sub>CN (gradient A: 10%-80%, 10 min), in 0.1% aqueous TFA at a flow rate of 1mL/min. The eluate was monitored by measuring the UV absorption at 214 nm. The system used was a Beckman System Gold.

### **FPLC analysis**

Ion exchange chromatography was carried out on a MonoQ Semi Prep column, eluted with a linear gradient of 500 mM NaCl (gradient B: 0%-100%, 60 min) in 50 mM TRIS/7M urea pH 8 at a flow rate of 2 min/mL, while monitoring the UV at 280 nm.

Reverse phase chromatography was carried out on a Pep RP Semi Prep 8494003 column, eluted with a linear gradient of 50 % CH<sub>3</sub>CN (gradient C: 0%-100%, 65 min, gradient D: 0%-100%, 120 min) in 0.05 M NH<sub>4</sub>HCO<sub>3</sub> at a flow rate of 2 mL/min, while monitoring the UV at 280 nm (gradient C) and at 214 nm (gradient D).

Reverse phase chromatography was also carried out on a Pep RPC 10/16 column, eluted with a linear gradient of 50 % CH<sub>3</sub>CN (gradient E: 10%-90%, 120 min, gradient F: 30%-100, 120 min), in 0.1 % TFA at a flow rate of 2 mL/min, while monitoring the UV at 214 nm.

The FPLC system used was a Pharmacia Biotech attached to a fraction collector Frac-100.

### **Mass spectrometry**

Mass spectral data was obtained using electrospray ionization (ESI) on a PE-Sciex API III Triple Quadrupole.

Mass spectral data was obtained using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) on a Bruker Biflex III spectrometer.

### **Sulfitolysis**

Sulfitolysis of the peptides were obtained by preparing a 10mg/mL solution of the particular peptide in 50mM TRIS/7 M urea pH 8, 100mM Na<sub>2</sub>SO<sub>3</sub> and 10mM Na<sub>2</sub>S<sub>4</sub>O<sub>6</sub>.2H<sub>2</sub>O. The reaction was left for one and a half hour at room temperature.

## **2.2. Synthesis of A-Chain Insulin Analogues**

0.285 g (0.2 mmol) of MBHA-Boc-Asp( $\alpha$ -Bzl) resin was placed in a standard 60 mL reaction vessel. The A-Chain  $D^{12}C(Acm)^{6,7,11}$  (**a**, molecular weight: 2624.99 g/mol) sequence was entered and run on a modified ABI430A peptide synthesizer using fast Boc-DEPBT single coupling. The peptide and resin (0.84 g) were transferred to a reaction vessel. The A-Chain analog was cleaved from the resin using HF in the presence of p-cresol (10 mL HF/1 mL p-cresol) in an ice bath for one hour. After HF removal, the residue was suspended in ether and filtered. Three different solutions were used to extract the peptide: TFA aqueous, 70% HCOOH and  $NH_4HCO_3$  pH 8.

### 2.3. Purification of A-Chain analogues: A-Chain $D^{12}C(Acm)^{6,7,11}C(SH)^{20}$ (**a**) and A-Chain $D^{12}C(Acm)^{6,7,11}C(SSO_3^-)^{20}$ (**b**)

#### Degradation studies

In order to purify the A-Chain analogues and to make chain combinations, it was first necessary to study the degradation of the peptide under basic conditions. 1 mg/mL solutions of the peptide in 0.1 M glycine at pH 8.5, 9.3 and 10.5 were left overnight at room temperature. The solutions were analyzed by HPLC (gradient A).

#### Purification of A-Chain $D^{12}C(Acm)^{6,7,11}$

A-Chain  $D^{12}C(Acm)^{6,7,11}C(SSO_3^-)^{20}$  (**b**, molecular weight: 2703.99 g/mol) was necessary to obtain, so sulfitolysis of 84 mg of the A-Chain  $D^{12}C(Acm)^{6,7,11}$  obtained after solid phase synthesis was carried out. The reaction mixture was purified by FPLC on a MonoQ Semi Prep column, using gradient B. The fractions containing the peptide were collected

and split in two equal volumes. One of those volumes was run on a RP Semi Prep column using gradient C. The fractions containing the product were collected and lyophilized. The product was analyzed by HPLC (gradient A).

The second volume was reacted in a 10 mM DTT solution for one hour at room temperature, in order to cleave the sulfite bond formed at Cys<sup>20</sup>. The solution was purified by FPLC on a Pep RP Semi Prep column using gradient C. The product was obtained after lyophilizing the collected fractions. Its HPLC chromatogram was analyzed using gradient A.

#### **2.4. Purification of B-Chain C(Acm)<sup>7</sup>C(SSO<sub>3</sub><sup>-</sup>)<sup>19</sup>(c)**

144 mg of a crude material of B-Chain C(Acm)<sup>7</sup>C(SSO<sub>3</sub><sup>-</sup>)<sup>19</sup> prepared by David Smiley at the DiMarchi laboratory, was sulfitolized.

The reaction mixture was purified by FPLC with a Pep RP Semi Prep column, using gradient D while monitoring the UV at 214 nm. The fractions containing the product were collected, lyophilized and analyzed by HPLC using gradient A.

#### **2.5. Synthesis of Insulin analogues using A-Chain D<sup>12</sup>C(Acm)<sup>6,7,11</sup>C(SH)<sup>20</sup>(a) and B-Chain C(Acm)<sup>7</sup>C(SSO<sub>3</sub><sup>-</sup>)<sup>19</sup>(c)**

**Formation of one interchain disulfide bond 1:1 molar ratio at room temperature, pH 8.5**

1 mg ( $3.8 \times 10^{-7}$  mol) of **a** was added to a solution of 1.4 mg (1 equiv) of **c** (molecular weight: 3581.14 g/mol) prepared by Jonathan Meyers at the DiMarchi laboratory, in 300  $\mu$ L 0.05 M  $\text{NH}_4\text{HCO}_3$  pH 8.5. The reaction mixture was left at room temperature and monitored by HPLC using gradient A after 1.5, 3 and 18 hours.

By FPLC, a Pep RP column was run, using gradient E. The solvent of the desired fractions was removed by lyophilization and analyzed by MALDI-TOF.

**Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 8.5**

4 mg ( $1.143 \times 10^{-6}$  mol) of **c** was dissolved in 900  $\mu$ L 0.05 M  $\text{NH}_4\text{HCO}_3$  pH 8.5 and added to 3 mg (1 equiv) of **a**. The reaction mixture was left at 4 °C and it was monitored by HPLC using gradient A after 1.5, 3 and 24 hours.

The reaction mixture was purified with a Pep RP column, using gradient F. The fractions containing the products were collected and lyophilized. The chromatogram of the product was analyzed using HPLC (gradient A).

In order to confirm the identity of the product 0.5 mg was treated by sulfitolysis. The solution was analyzed after one and half hour by HPLC (gradient A) and MALDI-TOF.

**Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 2.9, 6, 7.5 and 10**

The reactions were set up in the same way as the reactions made at room temperature. pH of 2.9 and 6 were obtained preparing 1% CH<sub>3</sub>COOH/20 % CH<sub>3</sub>CN solutions. The reactions were analyzed after 1.5 hours. The HPLC chromatograms were obtained using gradient A.

Reactions at pH 7.5 and 10 were prepared following the conditions already mentioned. The solutions were made using 0.05 M NH<sub>4</sub>HCO<sub>3</sub>. The reaction mixtures were left at 4 °C and monitored by HPLC using gradient A after 1.5, 3 and 24 hours.

**Formation of one interchain disulfide bond 1:2, 1:4 molar ratio at 4 °C, pH 7.5 and 8.5**

2.8 mg ( $7.63 \times 10^{-7}$  mol) of **c** were dissolved in 475 μL 0.05 M NH<sub>4</sub>HCO<sub>3</sub> pH 8.5. 1 mg (0.5 equiv) of **a** was added as a powder. The reaction mixture was left at 4 °C. The same procedure was followed to prepare the 0.05 M NH<sub>4</sub>HCO<sub>3</sub> pH 7.5 reaction solution.

5.5 mg ( $1.52 \times 10^{-6}$  mol) of **c** were dissolved in 812.5 μL 0.05 M NH<sub>4</sub>HCO<sub>3</sub> pH 8.5. 1 mg (0.25 equiv) of **a** was added as a powder. The reaction mixture was left at 4 °C. The same procedure was followed to prepare the 0.05 M NH<sub>4</sub>HCO<sub>3</sub> pH 7.5 reaction solution.

The reactions were monitored by HPLC using gradient A after 1.5, 3 and 22.5 hours.

**2.6. Synthesis of Insulin analogues using A-Chain  $D^{12}C(Acm)^{6,7,11}C(SSO_3^-)^{20}(b)$  and B-Chain  $C(Acm)^7C(SH)^{19}(d)$**

**Formation of one interchain disulfide bond 1:1 molar ratio at room temperature, pH 8.5**

1.3 mg ( $3.7 \times 10^{-7}$  mol) of **d** (molecular weight: 3502.14 g/mol) prepared by Jonathan Meyers was added to a solution of 1 mg (1 equiv.) of **b**, in 300  $\mu$ L 0.05 M  $NH_4HCO_3$  pH 8.5. The reaction mixture was left at room temperature and monitored by HPLC using gradient A after 1.5 and 3 hours.

**Formation of one interchain disulfide bond 1:1 and 2:1 molar ratio at 4 °C, pH 7.5 and 8.5**

1.3 mg ( $3.7 \times 10^{-7}$  mol) of **d** prepared by Jonathan Meyers at the DiMarchi laboratory, was added to a solution of 1 mg (1 equiv.) of **b**, in 300  $\mu$ L 0.05 M  $NH_4HCO_3$  pH 8.5. The reaction at pH 7.5 was prepared following the same procedure but using 300  $\mu$ L 0.05 M  $NH_4HCO_3$  pH 7.5. The reaction mixtures were left at 4 °C and monitored by HPLC using gradient A after 1.5, 3 and 24 hours.

1.3 mg ( $3.7 \times 10^{-7}$  mol) of **d** prepared by Jonathan Meyers at the DiMarchi laboratory, was added to a solution of 2 mg (1 equiv.) of **b**, in 440  $\mu$ L 0.05 M  $NH_4HCO_3$  pH 8.5. The same procedure was followed to prepare the solution at pH 7.5. The reactions were left at 4 °C and monitored by HPLC using gradient A after 1.5, 3 and 24 hours.



### 3. RESULTS AND DISCUSSION

#### 3.1. Synthesis of A-Chain Insulin Analogues

It was found that TFA dissolved the peptide without dissolving the resin. The 70% HCOOH solution needed the addition of pure HCOOH in order to dissolve the peptide entirely. Finally, the  $\text{NH}_4\text{HCO}_3$  pH 8 solution was able to dissolve the solid with the addition of  $\text{CH}_3\text{CN}$ .

HPLC analysis of the extracts were used to corroborate the presence of the A-Chain  $\text{D}^{12}\text{C}(\text{Acm})^{6,7,11}$  in the product. The peak located at 5.8 min in each chromatogram was confirmed to be the A-Chain desired by comparison with the HPLC chromatogram obtained from the same peptide synthesized by David Smiley (DLS-005-91, retention time: 5.85 min). The presence of the A-Chain analogue was also confirmed by mass spectrometry (ESI). The spectrum showed a peak located at 2623.0 of molecular weight (the expected mass was 2625).

The three extracts were lyophilized, and the white solids obtained were stored at 4 °C. TFA extract gave 88.5 mg, HCOOH extract gave 91.1 mg and  $\text{NH}_4\text{HCO}_3$  pH 8 extract gave 114 mg.

#### 3.2. Purification of A-Chain analogues: A-Chain $\text{D}^{12}\text{C}(\text{Acm})^{6,7,11}\text{C}(\text{SH})^{20}$ (a) and A-Chain $\text{D}^{12}\text{C}(\text{Acm})^{6,7,11}\text{C}(\text{SSO}_3^-)^{20}$ (b)

## Degradation studies

The chromatograms showed degradation of the peptide at pH 9.3 and 10.5 with the appearance of a second peak located at 5.6 min. Mass spectrometry (MALDI-TOF) analysis of the pH 8.5 solution after a 30 min exposure, was compared with the mass spectrum (MALDI-TOF) of the pH 10.5 solution left overnight. The latter spectrum did not show the peak of 2646.1096 of molecular weight which was considered to be from the peptide desired (the expected mass was 2625) and that appeared in the former spectrum.

A 6 hours study of a 1mg/mL solution of the peptide in 0.1 M glycine at pH 8.5 followed by HPLC analysis confirmed that at this particular pH, degradation did not occurred.

## Purification of A-Chain $D^{12}C(Acm)^{6,7,11}$

14 mg of a white solid was obtained of peptide **b** with a retention time of 5.35 min in its HPLC chromatogram, whereas 15.5 mg of a white product was obtained of peptide **a** with a retention time of 5.81 min in its chromatogram. The overall yield was 35 %.

### 3.3. Purification of B-Chain $C(Acm)^7C(SSO_3^-)^{19}(c)$

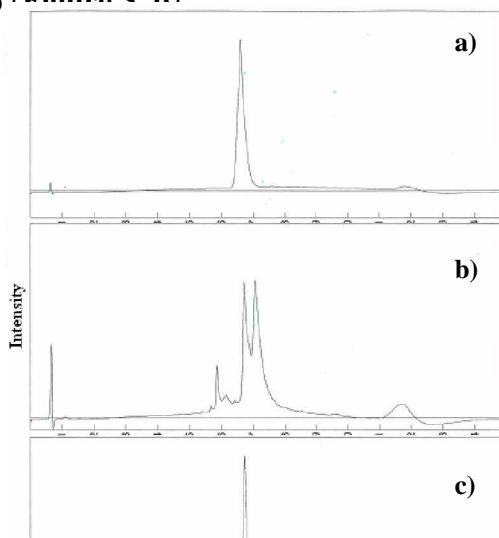
The HPLC chromatogram obtained from this product (30 mg, 21 % yield) confirmed the presence of the peptide. It had a retention time of 6.5 min which was the same as the one obtained with the B-Chain  $C(Acm)^7C(SSO_3^-)^{19}$  prepared by Jonathan Meyers at the DiMarchi laboratory.

### 3.4. Synthesis of Insulin analogues using A-Chain $D^{12}C(Acm)^{6,7,11}C(SH)^{20}$ (a) and B-Chain $C(Acm)^7C(SSO_3^-)^{19}$ (c)

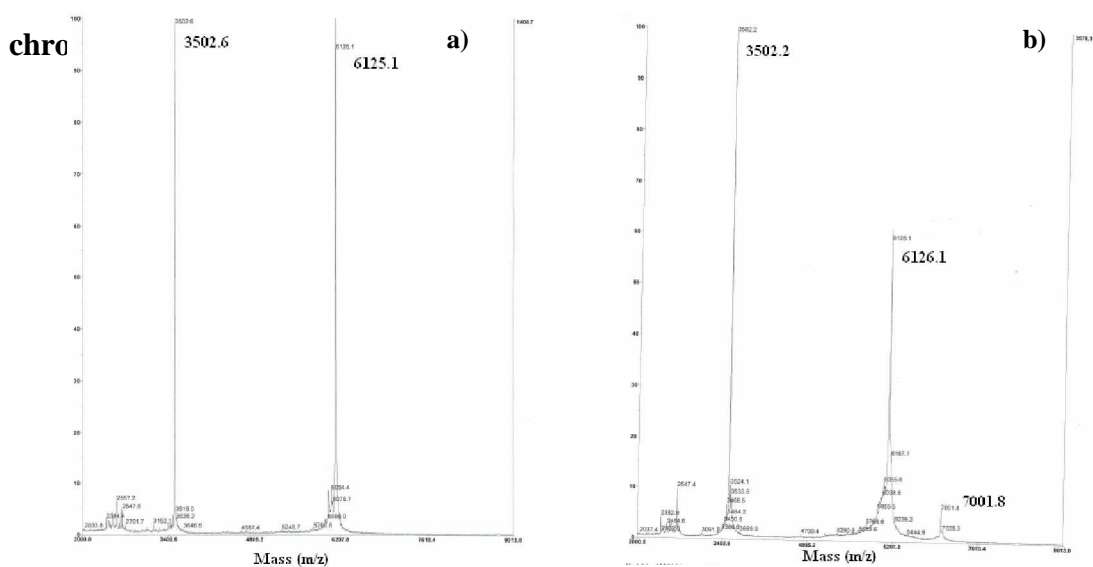
#### Formation of one interchain disulfide bond 1:1 molar ratio at room temperature, pH 8.5

The chromatograms showed the formation of two products with retention times of 6.68 and 7.1 min. By comparison with the HPLC analysis of a monodisulfide insulin synthesized by David Smiley (DLS-005-67B) (**Figure 2, a**) it was determined that the peak that appeared at 6.68 min corresponded to the desired product. MALDI-TOF analysis of the reaction after 3 hours confirmed the presence of the dipeptide. It showed a peak of M+1 of 6125.059 of molecular weight (the expected molecular weight was 6124).

Two fractions obtained during the purification of the reaction mixture, were analyzed by MALDI-TOF. It was found that the peak that appeared on analytical HPLC (gradient A) with retention time of 6.68 min (**Figure 2, c**) was effectively the dipeptide desired showing a molecular weight of M+1 of 6125.1 (**Figure 3, a**), whereas the peak that appeared at 7.1 min (**Figure 2, b**) was found to be a B-chain dimer with a molecular weight of M+1 of 7001.6 (**Figure 3, b**)



**Figure 2 a) Monodisulfide insulin synthesized by David Smiley (DLS-005-67B), b) Fraction ACI 20-2 from chromatography, c) Fraction ACI20-1 from**



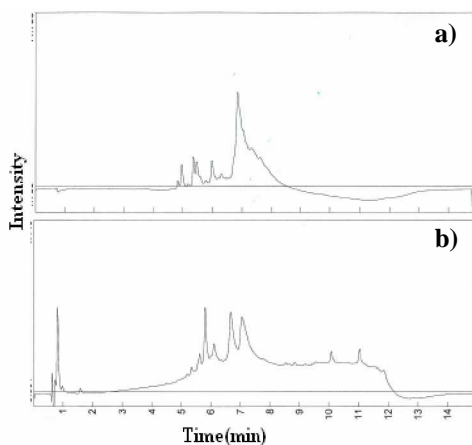
**Figure 3 a) Fraction ACI20-1 from chromatography, b) Fraction ACI 20-2 from chromatography.**

In the MALDI-TOF the B-Chain **d** was shown with molecular weight of M+1 of 3502.6. From this reaction less than one milligram was obtained which corresponds to less than 15 % yield.

With these results it was concluded that the active Cys(SH)<sup>20</sup> group of the A-Chain **a** attacked the interchain disulfide once formed releasing one molecule of B-chain containing an active thiol capable of react with the **c** peptide by thiolysis, leading to the formation of the B dimer. A reaction made at less temperature, in a more acidic solution, or using less amount of the active compound was thought to decrease the activity of the thiol group present at A20 accompanied by a decrease in the formation of the B dimer.

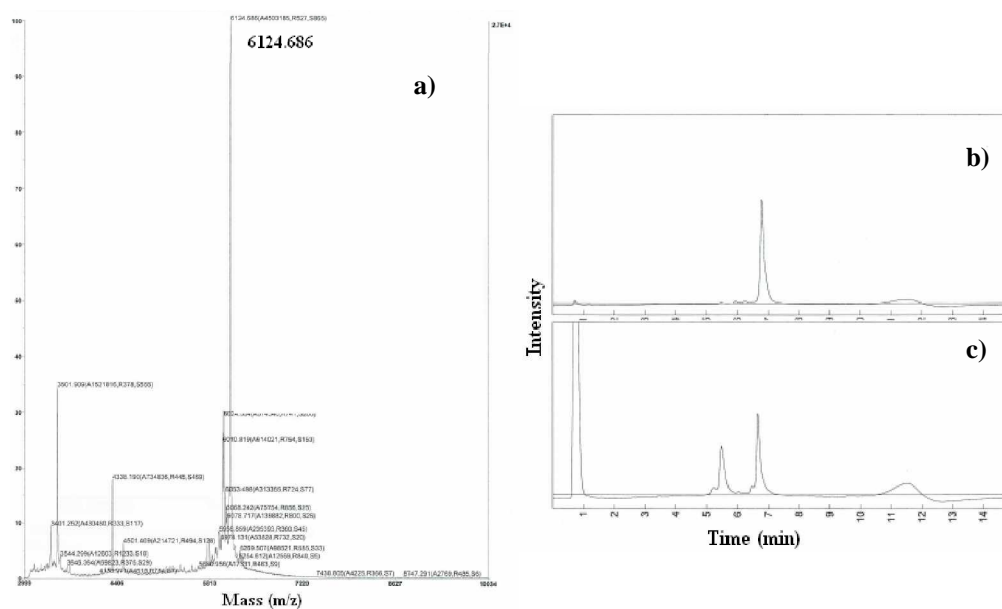
#### **Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 8.5**

From the chromatograms obtained it was found that the ratio between the peak of the dipeptide desired (6.7 min of retention time, **Figure 4, a**) and the one that corresponded to the B dimer was bigger than the same ratio observed when the reaction was made at room temperature (**Figure 4, b**). This confirmed that the nucleophilicity of the Cys(SH)<sup>20</sup> group found in the A-Chain was reduced at 4 °C.



**Figure 4 a) Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 8.5 after 18 h, b) Formation of one interchain disulfide bond 1:1 molar ratio at room temperature, pH 8.5 after 22 hours.**

The identity of the purified product was confirmed by MALDI-TOF (**Figure 5, a**) and by inspection of the chromatograms obtained after sulfitolysis. These showed the presence of **a** and **c**, which were separated from the monodisulfide insulin (**Figure 5, b and c**).



**Figure 5 a) MALDI-TOF of the purified product, b) HPLC chromatogram of the product, c) A-chain and B-chain separated after sulfitolysis of the product.**

In this reaction 1.1 mg was obtained which represented 20 % yield.

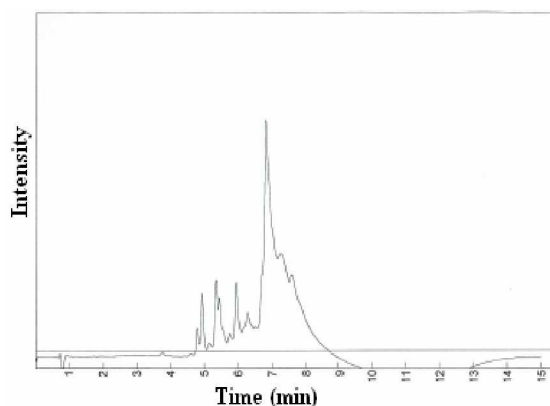
**Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 2.9, 6, 7.5 and 10**

At pH 2.9 peptide **b** could be dissolved but peptide **a** could not be dissolved, whereas at pH 6, **a** was soluble but **b** wasn't.

At pH 2.9 the only peak observed was the one that correspond to the B-Chain whereas at pH 6 just the peak corresponding to A-Chain was obtained.

From these results it was concluded that the lack of solubility of the reagents prevent the reaction take place.

From the HPLC chromatograms it was found that at pH 10 the reaction didn't take place. At pH 7.5 and after 24 hours the intensity of the peak that corresponds to the product desired (retention time of 6.8 min) was bigger than the one that was thought to be the B dimer (retention time of 7.2 min) (**Figure 6**) .



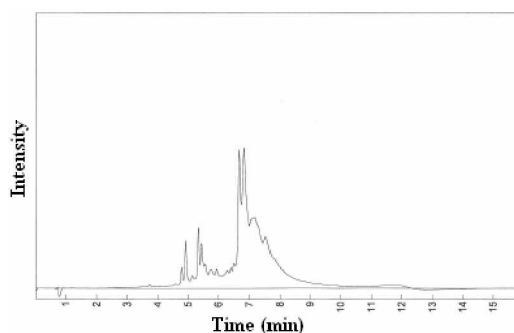
**Figure 6. Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 7.5.**

With these results it was confirmed that the nucleophilicity of the Cys(SH)<sup>20</sup> was reduced in a more acidic environment.

**Formation of one interchain disulfide bond 1:2, 1:4 molar ratio at 4 °C, pH 7.5 and 8.5**

From the chromatograms it can be concluded that the reaction using a:c/1:4, didn't give a considerable amount of product, neither at pH 7.5 nor at pH 8.5. After 22.5 hours it could be noted that the reaction did take place reflecting the slow rate of the reaction.

The best results were obtained using a:c/1:2 at pH 7.5 after 22.5 hours. However, the peak that corresponded to the dipeptide obtained (retention time of 6.8 min) had the same intensity as the peak that corresponded to the B-Chain (retention time of 6.6 min) (**Figure 7**).





**Figure 7. Formation of one interchain disulfide bond 1:2 molar ratio at 4 °C, pH 7.5**

**3.5. Synthesis of Insulin analogues using A-Chain  $D^{12}C(Acm)^{6,7,11}C(SSO_3^-)^{20}(b)$  and B-Chain  $C(Acm)^7C(SH)^{19}(d)$**

**Formation of one interchain disulfide bond 1:1 molar ratio at room temperature, pH 8.5**

The chromatograms showed three significant peaks. The peak that appeared at 6.8 min corresponded to the desired product. B-chain **d** (6.9 min) and B dimer (7.1 min) were also shown. Because the three peptides eluted with similar retention times the purification of the desired one could be a difficult step.

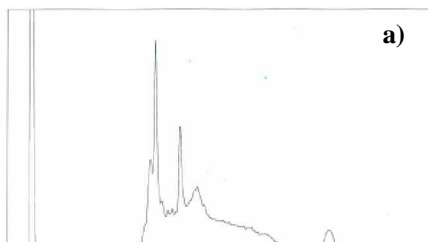
**Formation of one interchain disulfide bond 1:1 molar ratio at 4 °C, pH 7.5 and 8.5**

The chromatograms showed that a considerable amount of product did not form under these conditions.

**Formation of one interchain disulfide bond 1:1 and 2:1 molar ratio at 4 °C, pH 7.5 and 8.5**

The chromatograms showed that using **b:d**/2:1 molar ratio more product was obtained (in comparison with the starting materials) than using a 1:1 molar ratio.

At pH 8.5 (2:1 molar ratio) it was obtained a greater amount of product (retention time of 6.6 min) in comparison with the one obtained at pH 7.5 (**Figure 8**).



**Figure 8 a) Formation of one interchain disulfide bond 2:1 molar ratio at 4 °C, pH 8.5, after 3 hours b) Formation of one interchain disulfide bond 2:1 molar ratio at 4 °C, pH 7.5, after 4 hours.**

#### **4. CONCLUSIONS**

Extraction of the peptide using a  $\text{NH}_4\text{HCO}_3$  pH 8 solution was found to give good amounts of crude A-chain after its solid phase synthesis.

A-chain degrades at pH 9.3 and 10.5 but it remains stable on a pH 8.5 0.1 M glycine solution.

The procedures used to purify the A-Chain and B-Chain after their chemical synthesis were successful. The purity of the peptides was confirmed by HPLC and Mass Spectrometry analysis.

The formation of the interchain disulfide bond was successfully obtained using step 4 from **Scheme 1**. The identity of the peptide was confirmed by HPLC and Mass Spectrometry analysis.

In the formation of the interchain disulfide bond between A-Chain  $D^{12}C(Acm)^{6,7,11}C(SH)^{20}$  and B-Chain  $C(Acm)^7C(SSO_3^-)^{19}$  it was found that the reaction needs to take place at 4 °C and in a slightly acidic solution in order to decrease the activity of the thiol group present at A20 that cause the formation of B dimer. By inspection of the data it could be obtained a better yield making the reaction at pH 7.5 with a 1:1 molar ratio of the peptides at 4 °C. It is also important to mention that the reaction does not take place at pH 2.9 nor at pH 6 due to the lack of solubility of the peptides.

In the formation of the interchain disulfide bond between A-Chain  $D^{12}C(Acm)^{6,7,11}C(SSO_3^-)^{20}$  and B-Chain  $C(Acm)^7C(SH)^{19}$  it was found that making the reaction at 2:1 molar ratio, 4 °C and pH 8.5 gave a product with less amount of side products.

Finally, among all the conditions, the reaction make between A-Chain  $D^{12}C(Acm)^{6,7,11}C(SH)^{20}$  and B-Chain  $C(Acm)^7C(SSO_3^-)^{19}$  on a pH 7.5 solution with a 1:1 molar ratio of the peptides at 4 °C was found to be the best option.

## 5. FUTURE WORK

A new reaction between A-Chain  $D^{12}C(Acm)^{6,7,11}C(SH)^{20}$  and B-Chain  $C(Acm)^7C(SSO_3^-)^{19}$  using the set of conditions found should be carried out. The product should be purified and characterized by HPLC and Mass Spectrometry analysis. The amount of product should be quantified in order to determine the overall yield. If the yield obtained is good, a large scale reaction should be carried out in order to proceed with step 5 in the process shown in **Scheme 1**.

A new set of reactions should be carried out in which the overall concentration of the peptides should be decrease in order to determine its effects in the formation of the dipeptide. This could decrease the amount of side products obtain during the reaction.

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