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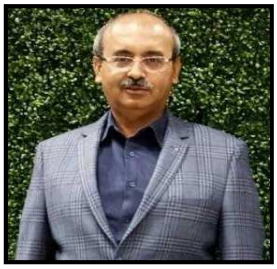

IMPACT OF PURIFICATION OF FMOC-AMINO ACIDS ON PEPTIDE PURITY IN SOLID PHASE PEPTIDE SYNTHESIS

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ABSTRACT

Solid Phase Peptide Synthesis, based on FMOC-chemistry is currently an extremely preferred approach in chemical synthesis of Peptides though it has various limitations attributed by high raw material costs, solvents and waste volumes[1]. Downstream processing steps of peptides involves coupling of several amino acids, each amino acid have contained considerable proportions of impurities. High process impurities and solvent load intensifies the complexity of conventional purification approaches. In our study, we employed raw material purification prior to peptide synthesis by which we aimed to bring down the residual impurity level of peptide under the study and reduction of waste load on HPLC which lead to higher peptide purity. A comparison of crude peptide and the peptide synthesized with purified Amino acids showed >15% increase in purity.

KEYWORDS

Fmoc-amino acids, Raw material, Recrystallization, Preparative HPLC, Peptide purity and Solid phase peptide synthesis.

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INTRODUCTION

Peptides are amino acid polymers with wide-structural diversity and various biological functionalities[2]. Peptides can be obtained mainly through Solid Phase Chemical Synthesis, Liquid Phase Chemical Synthesis and from natural sources[3]. Presently, peptides are being used in various applications such as drug manufacturing[4], cosmetics[5] and food processing industry.

Peptides represent an excellent starting point for design of novel therapeutics due to their intrinsic properties and pharmacological profile[6]. Currently, there are over 60 US-FDA approved peptide drugs in the market[7] and peptides continue to claim their importance in clinical development with approximately 140 peptide drugs currently in clinical trials and more than 500 therapeutic peptides in pre-clinical development.

While variety of methods are now available for commercial-scale manufacture of peptides, chemical synthesis is still the most widely used as it allows coupling of all possible sequences, including those containing unnatural amino acids or amino acids that are not entirely synthetic in origin[8]. Solid-phase Chemical Synthesis method is favorably used for synthesis of peptides in research laboratories and pharmaceutical industry[9].

Solid-Phase Peptide Synthesis consists of consumption of huge amounts of hazardous reagents and solvents[10]. Solvents represent the vast majority of the waste generated in solid-phase peptide synthesis. The main solvents used for solid-phase peptide synthesis are Dimethylformamide, N-Methyl-2-Pyrrolidone, and in lower amounts, dichloromethane, diethyl ether, and *tert*-butyl methyl ether. All these solvents present various hazards[11].

About 10-50 amino acids' coupling and deprotection steps are performed in each synthesis of peptides. The overall crude purity usually obtained is ~20-40% only. Residual impurities present in each amino acid used for coupling impacts the overall impurity of the peptide.

Amino acid deletion may cause truncated peptide impurities[12]; charging of excess of Fmoc-amino acids, while may ensure efficient coupling may sometimes cause insertion of additional amino acid into the desired peptide sequence[13]. Incomplete removal of permanent protecting groups after Solid Phase Peptide Synthesis may cause covalently attached protecting group-peptide impurities[14]. Certain amino acids tend to experience oxidations or reduction during solid-phase peptide synthesisprocedure[15]. Racemization of amino acids may cause diastereoisomeric impurities[16]. Aggregation of peptides during coupling steps may cause formation of dimer impurities[17].

Purification of peptides derived from solid-phase peptide synthesis requires the removal of deletion peptides resulting from incomplete coupling/deprotection steps, from racemization or side-chain rearrangement, and from various chemical substances introduced during the deprotection or cleavage stages of solid-phase peptide synthesis. Thus, there is a requirement of an efficient purification method of peptides to be employed after completion of solid-phase peptide synthesis.

Glucagon (Figure No.1) is a 29 amino acid polypeptide synthesized mainly in pancreatic α-cells following cleavage of proglucagon. It is a hormone with a molecular weight of about 3485 Da. The ratio of insulin to glucagon is normally tightly regulated. In healthy humans, absorption of nutrients from food stimulates insulin release and decreases glucagon levels, while hypoglycemia inhibits insulin and stimulates glucagon secretion. In patients with type 2 diabetes, insulin secretion is often delayed and reduced, together with defects in insulin action, while glucagon levels remain unchanged or elevated.

Patients with type 2 diabetes exhibit increased levels of fasting blood glucose, insulin resistance, reduced glucose stimulated insulin release, and progressive β-cell failure[18]. Administration of Glucagon or Glucagon- like peptide drugs have shown to consistently improve Glucose tolerance and glycemic control in patients with Diabetes mellitus Type 2.

High-Performance Liquid Chromatography is an advanced technique of column liquid chromatography and is a popular analytical technique used for the separation, identification and quantification of each constituent of a mixture. It is used for analysis of various compounds like synthetic drugs, biomolecules and natural compounds, its important application being isolation of individual constituents from a mixture of compounds.

HPLC is a rapid and efficient separation method which facilitates employment of phase systems that

do not cause excessive degradation of proteins[19]. Its preparative and process applications are undergoing rapid development for isolation and purification of peptides[20]. Preparative HPLC is used in most industries in place of Preparative column chromatography, but the cost of preparative HPLC column is very high.

In our study, we performed purification of every single amino acid used in the synthesis of Glucagon, to eliminate the residual impurity present in the Fmoc-amino acids that impact the overall crude purity. Further we attempted elevation of purity of crude peptide by subjecting it to additional purification methods such as Preparative HPLC.

MATERIAL AND METHODS

Reagents and chemicals

Experimental Section

Unless stated, all reagents and solvents used in this study were commercially available. During development, reactions were monitored by Kaiser Test and HPLC.

METHODOLOGY

Experimental Section

Purification of individual Fmoc-amino acids

Purification of Fmoc-Lys (Boc)-OH

Charge Fmoc-Lys (Boc)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (98g).

Purification of Fmoc-Thr (tBu)-OH

Charge Fmoc-Thr (tBu)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (71.3g).

Purification of Fmoc-Gln (Trt)-OH

Charge Fmoc-Gln (Trt)-OH (100g) in a flask, followed by Isopropyl Alcohol (500ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Isopropyl Alcohol. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (97g).

Purification of Fmoc-Ser (tBu)-OH

Charge Fmoc-Ser (tBu)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (74g).

Purification of Asp (OtBu)-OH

Charge Fmoc-Asp (OtBu)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid.

Purification of Fmoc-Asn (Trt)-OH

Charge Fmoc-Asn (Trt)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (98g).

Purification of Fmoc-Met-OH

Charge Fmoc-Met-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (98g).

Purification of Fmoc-Phe-OH

Charge Fmoc-Phe-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (98g).

Purification of Fmoc-Tyr (tBu)-OH

Charge Fmoc-Tyr (tBu)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (98g).

Purification of Fmoc-Ala-OH

Charge Fmoc-Ala-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (92g).

Purification of Fmoc-Gly-OH

Charge Fmoc-Gly-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (99.4g).

Purification of Fmoc-Arg (Pbf)-OH

Charge Fmoc-Arg (Pbf)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (99g).

Purification of Fmoc-Val-OH

Charge Fmoc-Val-OH (100 g) in a flask, followed by Toluene (800ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (97g).

Purification of Fmoc-Leu-OH

Charge Fmoc-Leu-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (96g).

Purification of Fmoc-Trp (Boc)-OH

Charge Fmoc-Trp (Boc)-OH (100g) in a flask, followed by Isopropyl Ether (200ml). Stir the mixture for 30 minutes. Charge n-Heptane (1500ml) in another flask to which the above IPE-Fmoc-Trp (Boc)-OH mixture was transferred to. Stir well the contents for about 1 hour. Filter and wash with n-Heptane. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (86g).

Purification of Boc-His (Trt)-OH

Charge Boc-His (Trt)-OH (100g) in a flask, followed by Toluene (600ml). Raise the temperature of reaction mass to 50°C which is stirred and maintained for 1 hour. Bring down the temperature to 30±5°C and keep stirring for about 2 hours. Filter and wash with Toluene. Collect the wet cake and dry under vacuum at 50°C to obtain purified amino acid (44g).

These amino acids are used for the preparation of the peptide drug, Glucagon (Scheme No.1).

Stage-I: Preparation of Resin protected Glucagon (GLU-I)

Wang Resin (25g) was swelled in Dichloromethane at ambient temperature for 30 ± 15 min in a Solid phase peptide synthesizer vessel, then it is reacted with Fmoc-Thr (tBu)-OH (12.81g) and Diisopropyl carbodiimide (5.02ml) in the presence of Dimethyl amino pyridine (0.552g). After achieving the UV

loading it is reacted with acetic anhydride (13.5ml) and pyridine (13.5ml) to protect unreacted Wang resin. Deprotect the Fmoc group using Piperidine: Dimethyl formamide mixture (200ml) and Couple with Fmoc-Asn (Trt)-OH/Oxymapure (4.58g)/ Diisopropyl carbodiimide (5.02ml) mixture after completion of reaction follow Fmoc deprotection and Coupling step for following amino acids as per the sequence:

Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Trp (Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Asp (OtBu)-OH, Fmoc-Gln (Trt)-OH, Fmoc-Ala-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Ser (tBu)-OH, Fmoc-Asp (OtBu)-OH, Fmoc-Leu-OH, Fmoc-Tyr (tBu)-OH, Fmoc-Lys (Boc)-OH, Fmoc-Ser (tBu)-OH, Fmoc-Tyr (tBu)-OH, Fmoc-Asp (OtBu)-OH, Fmoc-Ser (tBu)-OH, Fmoc-Thr (tBu)-OH, Fmoc-Phe-OH, Fmoc-Thr (tBu)-OH, Fmoc-Gly-OH, Fmoc-Gln (Trt)-OH, Fmoc-Ser (tBu)-OH, Boc-His (Trt)-OH.

After completion of couplings Wash the resin with N, N-dimethyl formamide, dichloromethane, Methanol and tert-butyl methyl ether then dry the resin under vacuum to get Resin protected Glucagon (GLU-I).

Stage-II: Preparation of Crude Glucagon (GLU-II)

In a flask, charge Tri-Fluoro Acetic Acid (32ml). Cool it down to 0°C. Slowly add water (2ml), Triisopropyl Silane (2ml), 2, 2′-(Ethylenedioxy) diethanethiol (4ml) and phenol (4g). Charge Resin-protected Glucagon (GLU-I) (8g), Stir the reaction mixture for about 40 minutes after which slowly raise the reaction temperature to 25°C. Stir and maintain the temperature for 3 hours. Filter the reaction mixture and wash with Tri-Fluoro Acetic Acid (10ml).

In another flask, charge methyl tert-butyl ether (320ml). Slowly add above filtrate mass after which stir and maintain reaction mixture temperature (0-5°C) for about 2 hours. Filter the solid. Wash the solid with tert-butyl methyl ether (100ml) followed by ethyl acetate (100ml). Dry the solid under vacuum at 35°C to obtain Crude Glucagon (GLU-II).

Stage-III: Preparation of Glucagon (GLU-III)

Crude Glucagon (GLU-II) is purified by preparative HPLC using C 18 ODS silica, TFA, water, acetonitrile then Ammonium bicarbonate, Water and acetonitrile buffers as eluants. After collection of all fractions, freeze dry the product meeting specification fractions to get Glucagon (GLU-III).

RESULTS AND DISCUSSION

Initial percentage of impurities present in Fmoc-amino acids used in the synthesis of Glucagon (29-amino acid chain) was ~26%, which was brought down to ~10% by purification of every Fmoc-amino acid used for Glucagon synthesis. The impurity levels in the crude/commercial and purified Fmoc-amino acids are given in Tables No.1 and Table No.2.

Purity of Glucagon synthesized with purified amino acids is 68.08, which is 15% more than Glucagon synthesized with raw materials not subjected to prior purification.

This was then used for preparation of Glucagon and the HPLC purity of the same is depicted in Figure No.4.

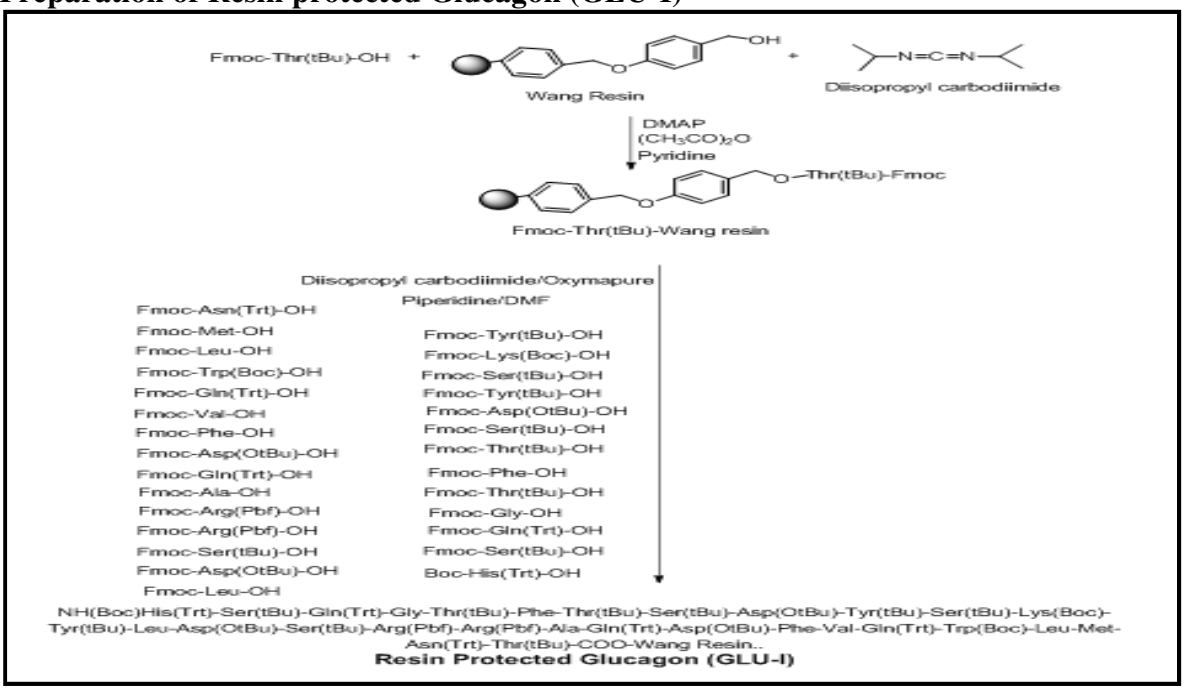
Table No.1: HPLC Purity data of commercial Fmoc-amino acids

S.No	Name of Amino Acid	HPLC Purity (%)	Impurity (%)	Number Times amino acid used in peptide Seq	Total Impurity
1	Fmoc-Lys (Boc)-OH	99.58	0.42	1	0.42
2	Fmoc-Thr (tBu)-OH	99.75	0.25	3	0.75
3	Fmoc-Gln (Trt)-OH	99.39	0.61	3	1.83
4	Fmoc-Ser (tBu)-OH	99.21	0.79	4	3.16
5	Fmoc-Asp (OtBu)-OH	99.57	0.43	3	1.29
6	Fmoc-Asn (Trt)-OH	98.7	1.3	1	1.3
7	Fmoc-Met-OH	99.53	0.47	1	0.47
8	Fmoc-Phe-OH	99.03	0.97	2	1.94
9	Fmoc-Tyr(tBu)-OH	98.78	1.22	2	2.44
10	Fmoc-Ala-OH	99.69	0.31	1	0.31
11	Fmoc-Gly-OH	99.27	0.73	1	0.73
12	Fmoc-Arg (Pbf)-OH	97.5	2.5	2	5
13	Fmoc-Val-OH	98.88	1.12	1	1.12
14	Fmoc-Leu-OH	99.62	0.38	2	0.76
15	Fmoc-Trp (Boc)-OH	96.71	3.29	1	3.29
16	Boc-His (Trt)-OH	98.99	1.01	1	1.01
17	Total	---	---	29	25.82

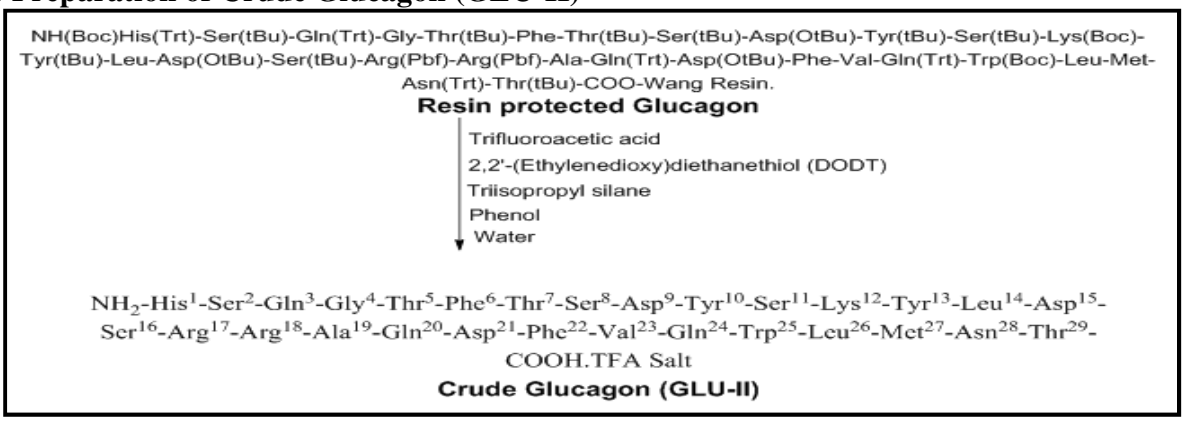
Table No.2: HPLC Purity data of purified Fmoc-amino acids

S.No	Name of Amino Acid	HPLC Purity (%)	Impurity (%)	Number Times amino acid used in peptide Seq	Total Impurity
1	Fmoc-Lys (Boc)-OH	100	0	1	0
2	Fmoc-Thr (tBu)-OH	100	0	3	0
3	Fmoc-Gln (Trt)-OH	99.79	0.21	3	0.63
4	Fmoc-Ser (tBu)-OH	99.95	0.05	4	0.2
5	Fmoc-Asp (OtBu)-OH	99.76	0.24	3	0.72
6	Fmoc-Asn (Trt)-OH	98.97	1.03	1	1.03
7	Fmoc-Met-OH	99.71	0.29	1	0.29
8	Fmoc-Phe-OH	100	0	2	0
9	Fmoc-Tyr (tBu)-OH	99.51	0.49	2	0.98
10	Fmoc-Ala-OH	99.15	0.85	1	0.85
11	Fmoc-Gly-OH	99.37	0.63	1	0.63
12	Fmoc-Arg (Pbf)-OH	99.48	0.52	2	1.04
13	Fmoc-Val-OH	99.62	0.38	1	0.38
14	Fmoc-Leu-OH	100	0	2	0
15	Fmoc-Trp (Boc)-OH	96.41	3.59	1	3.59
16	Boc-His (Trt)-OH	99.69	0.31	1	0.31
17	Total			29	10.65

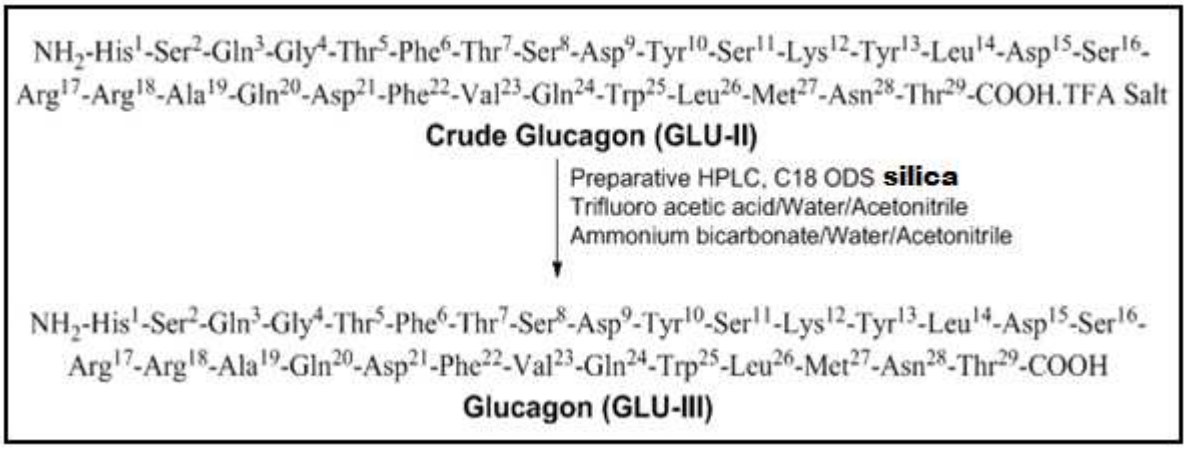
Stage-I: Preparation of Resin protected Glucagon (GLU-I)



Stage-II: Preparation of Crude Glucagon (GLU-II)



Stage-III: Preparation of Glucagon (GLU-III)



Scheme No.1 (Thee stages): Synthetic scheme for preparation of glucagon

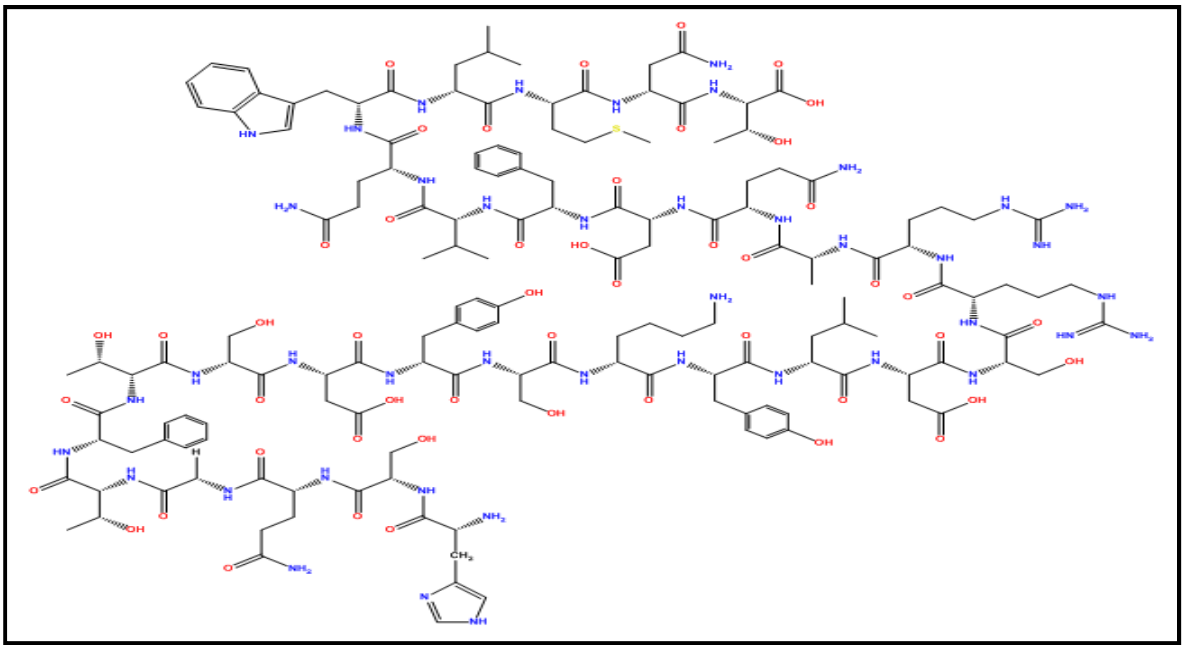


Figure No.1: Structure of Glucagon

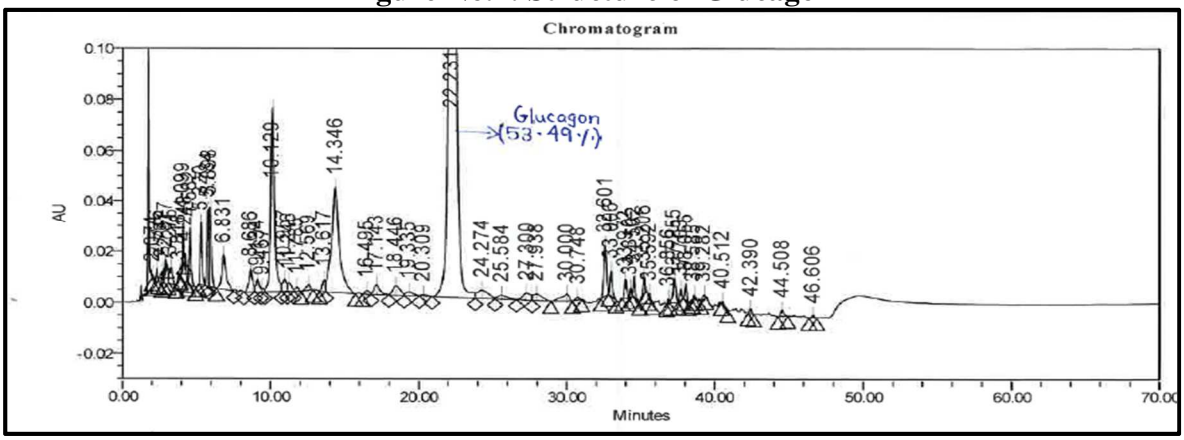


Figure No.2: Chromatogram of Crude Glucagon (GLU-II) synthesized with crude Fmoc-amino acids; Purity - 53.49%

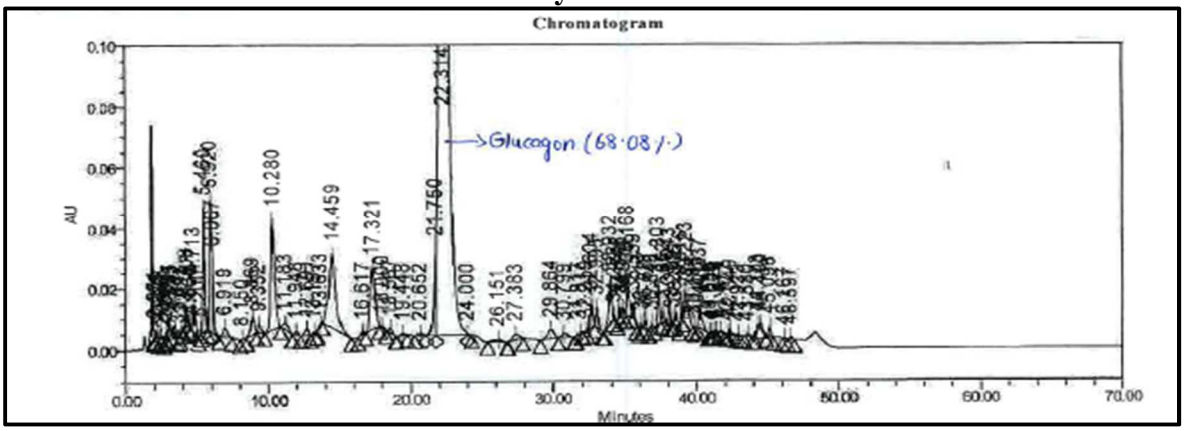


Figure No.3: Chromatogram of Crude Glucagon (GLU-II) synthesized with purified Fmoc-amino acids; Purity - 68.08%

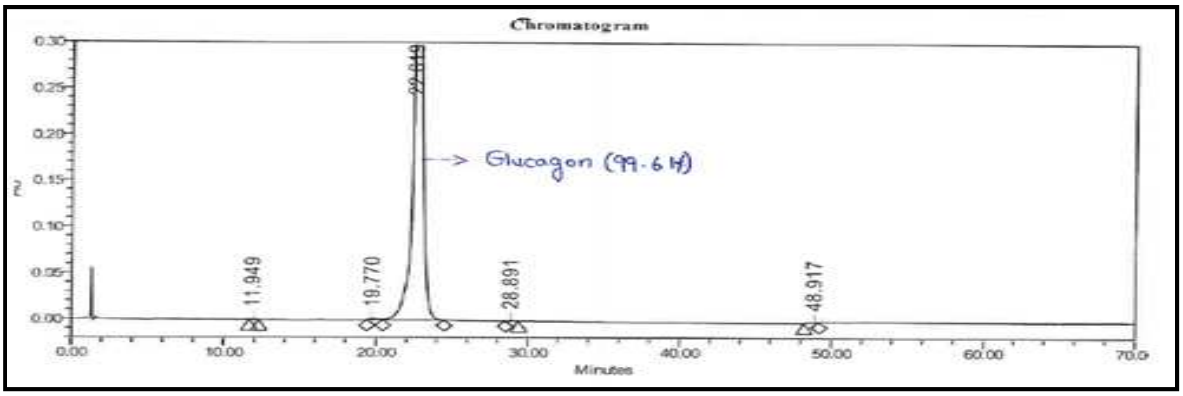


Figure No.4: HPLC purity of Glucagon (GLU-III); Purity - 99.61% and all unknown impurities < 0.1 %

CONCLUSION

It can be determined from the above study that, by purifying amino acids before synthesizing our target peptide, we could also achieve reduction of impurities contributed by raw materials from ~25% to ~10%. Some significant impurities were completely removed due to this prior purification. This had a positive impact on the elevation of peptide purity and decreasing the sample load of Preparative HPLC, leading to faster and better purification of the final product with all unknown impurities less than 0.1%.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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