Optimization of Transpeptidation Reaction of the Insulin Precursor for Efficient Yield Recovery during Downstream Processing

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Submitted by

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DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Optimization of Transpeptidation Reaction of the Insulin Precursor for Efficient Yield Recovery during Downstream Processing.''** submitted by **the undersigned,** has been carried out under the supervision of Professor Dr. Naiyyum Choudhury Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka and Dr. Iqbal Hasan Khan, General Manager Research & Development,Biotech Division Incepta Pharmaceutical. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Syed Morsalin Ali

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ABBREVIATIONS

AOX	Alcohol Oxidase
ATP	Adenosine Triphosphate
Conc.	Concentration
DNA	Deoxyribo Nuclic Acid
DMF	Dimethylformamide
DM	Diabetes Mellitus
EtOH	Ethanol
Fig	Figure
GDM	Gestational Diabetes Mellitus
GAA	Glacial Acetic Acid
gm	Gram
HPLC	High Performance Liquid Chromatography
hr.	Hour
IP	Insulin Precursor
mM	Milli mole
ml	Millilitter
MRL	Molecular Research Lab
O-Thr ester	O-tert-Butyl-L-threonine –tert-butyl ester
PTMs	Post-translational Modifications
PC	Prohormoneconvertases
RPC	Reversed-phase chromatography
Temp.	Temperature

TFA	Trifluoroacetic acid
WHO	World Health Organization
w/w	weight/weight

ABSTRACT

Nowadays Yeast cell is a popular host for recombinant human insulin production where initially it secrets insulin precursor fusion protein with N-terminal spacer peptide and deletedthreonineB30, followed by a small C-peptide connected with its A andB chains. The insulin precursor is then purified and subsequently converted into human insulin ester via a slow transpeptidation reaction (hydrolysis and coupling) in presence of both trypsin and O-tert-Butyl-L-threonine –tert-butyl ester (O-Thr-ester). Transpeptidation reaction is very critical for recovery of insulin at the least expenses of the aforementioned chemicals. In this study a protocol has been developed where certain parameters have been changed in congruence with published data. The major focus was to use low amount of trypsin and O-Thr-ester for the transpeptidation reaction as well as changing organic solvent composition, water content, pH, time and temperature. In this study, a two-step transpeptidation reaction has been proposed instead of one-step reaction process by separating the cleavage step from the coupling step so that each reaction was performed under its optimal condition. Through this method, the total the conversion of insulin ester increased 57.44% and the reaction time was reduced 58.33% by using the same amount of trypsin and O-Thr-ester compared with the one-step method and available published data. Thus, this two-step transpeptidation strategy was simple, efficient, suitable for scale-up and cost effective and could be used for the pharmaceutical production of human insulin.

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Chapter 1

Introduction

1.1 General overview of Insulin Production:

Diabetes mellitus is a chronic metabolic disease characterized by hyperglycemia. It's also one of the most common diseases worldwide and continues to increase in number, as economic development and urbanization results in changing lifestyles that are characterized by reduced physical activity and increased obesity.[1] Insulin is the first pharmaceutical protein for the treatment of diabetes mellitus, it has been in use for over 90 years. Since early eighties *Escherichia coli* and *Saccharomyces cerevisiae* have been successfully used for the production of recombinant human insulin by both Eli Lilly and Novo Nordisk.[2.] The therapeutic insulin remains one of the most commonly used products of pharmaceutical biotechnology and insulin-based products now account for more than 30% of the entire diabetes medication market share. In general, *Pichia pastoris* has been developed into one of the most widely used expression systems for the production of heterologous proteins because it combines the ease of genetic manipulation with rapid growth on an inexpensive medium, resulting in high cell densities and has the capability for complex posttranslational modifications. Moreover, the recombinant products are free of endotoxins, as well as oncogenic and viral DNA.[3–5] In recent years, *P. pastoris* has been reported as an important alternative yeast species for insulin production.[6–8] The expression of human proinsulin fused to the α-mating factor leader in *S. cerevisiae* did not result in the efficient secretion of proinsulin or insulin into the culture supernatant.[9] However, the expression of a cDNA encoding a proinsulin-like molecule with the deletion of threonineB30, followed either by the replacement of the human proinsulin C-peptide with a small C-peptide (RR, AAK, or EWK) or by direct fusion of lysineB29 to glycineA1 and the subsequent fusion to the α-factor leader, resulted in the efficient expression of single-chain proinsulin-like molecules.[10,11] Using this strategy, a recombinant *P. pastoris* strain that could efficiently express the single-chain insulin precursor, consisting of the first 29 amino acids of the human insulin B chain (B1–B29), linked to 21 amino acids of the human insulin A chain (A1–A21) via the mini C-peptide AAK connecting to lysineB29 and glycineA2.[12] In this strain, a spacer peptide (EEAEAEAEPK) placed between the leader peptide (signal peptide) and insulin precursor was inserted to improve Kex2 endoprotease processing and insulin precursor fermentation yield. A high production of insulin precursor fusion protein, 3.6 g/L of cell-

free culture supernatant, by *P. pastoris* in the microbial fermentor was achieved.[8] The secreted single-chain insulin precursor fusion protein could then be purified and subsequently converted into human insulin ester (ThreonineB30[tBu]-OtBu human insulin) via one-step tryptic transpeptidation in an organic aqueous medium in the presence of trypsin and O-tert-Butyl-L-threonine –tert-butyl ester (O-Thr-ester).[12]

The insulin precursor fusion protein was transformed into human insulin ester via one-step enzymatic transpeptidation in the presence of high concentrations of trypsin and H-Thr(tBu)-OtBu to ensure a high conversion ratio.[13]

1.1.1 Recombinant Human Insulin production by *E. coli* expression system:

Recombinant human insulin was first produced in *E. coli* by Genentech in 1978, using a approach that required the expression of chemically synthesized cDNA encoding for the insulin A and B chains separately in *E. coli*.[14] After expressing independently, the two chains are purified and co-incubated under optimum reaction conditions that promoted the generation of intact and bioactive insulin by disulphide bond formation. The first commercial recombinant insulin was developed for therapeutic use in human by this two-chain combination procedure.[15] Another approach involves the expression of a single chemically synthesized cDNA encoding for human proinsulin in *E. coli* followed by purification and subsequent excision of C-peptide by proteolytic digestion. This approach was more efficient and convenient for large scale production of therapeutic insulin as compared to the two followed this technology to produce Humulin, the first recombinant insulin approved in 1982, for the treatment of diabetic patients. These first generation recombinant insulins have an amino acid sequence identical to native human insulin and are preferred over animal derived insulin products.[16] However, advancement in the field of genetic engineering and development of technology chain combination approach and has been used commercially since 1986.[15]

E. coli is a preferred microorganism for large-scale production of recombinant proteins. However, several disadvantages limit its use for production of recombinant biopharmaceuticals. Various post-translational modifications (PTMs) such as glycosylation, phosphorylation, proteolytic processing and formations of disulfide bonds which are very crucial for biological activity, do not occur in *E. coli*.[17]

1.1.2 Yeast expression system for the production of insulin:

Now a day's Yeast is a preferred host for expression of various heterologous proteins that require post-translational modifications for its biological activity. Yeast cell has the ability to carry out numerous post-translational modifications such as phosphorylation, O-linked glycosylation, N-linked glycosylation, acetylation and acylation. Recombinant proteins are expressed in soluble form in yeast and properly folded in functionally active form. Production of biopharmaceuticals using yeast expression system is also very cost effective and is amenable to scale up using large bioreactors. However, one major concern for producing therapeutic glycoprotein for human application is that yeast N-glycosylation is of the high-mannose type, which confers a short half-life in vivo and hyper–immunogenicity and thus render the therapeutic glycoprotein less effective. Various attempts have been made to humanize yeast N-glycosylation pathways in order to produce therapeutic glycoproteins with humanized N-glycosylation structure.[18] Like *E. coli*, yeast derived recombinant biopharmaceuticals majorly intended as therapeutics for infectious diseases or endocrine, metabolic disorders. Alternate yeast strains, besides *S. cerevisiae*, are being explored for large-scale production of biopharmaceuticals. Specifically, *Pichia pastoris* has the ability to attain high cell densities by its robust methanol-inducible alcohol oxidase 1 (AOX1) promoter and simple developmental approaches contribute to high quality and quantity of recombinant proteins production. In comparison to *Saccharomyces cerevisiae, Pichia pastoris* provides a major advantage in the glycosylation of secreted proteins because it does not hyperglycosylate the heterologous proteins. Both yeast strains have a majority of N-linked glycosylation of the high-mannose type, but the length of the oligosaccharides chain added to proteins in Pichia (around 8–14 mannose residues per side chain) is much shorter than those expressed in Saccharomyces cerevisiae (approximately 50–150 mannose residues per side chain), suggesting that glycoproteins produced in *Pichia pastoris* may be more suitable for therapeutic use in humans.[19] Moreover, very high level of expression of heterologous proteins can be attained in *P. pastoris*,that might constitute about 30% of total cellular protein which is very high as compared to *S. cerevisiae*.[20] Therefore, *P. pastoris* can be an attractive alternate for large-scale production of recombinant insulin and insulin analogues. Comparing the different insulin production systems where the

bacterial expression systems show higher average specific productivity and maximum biomass concentrations are higher in yeast.

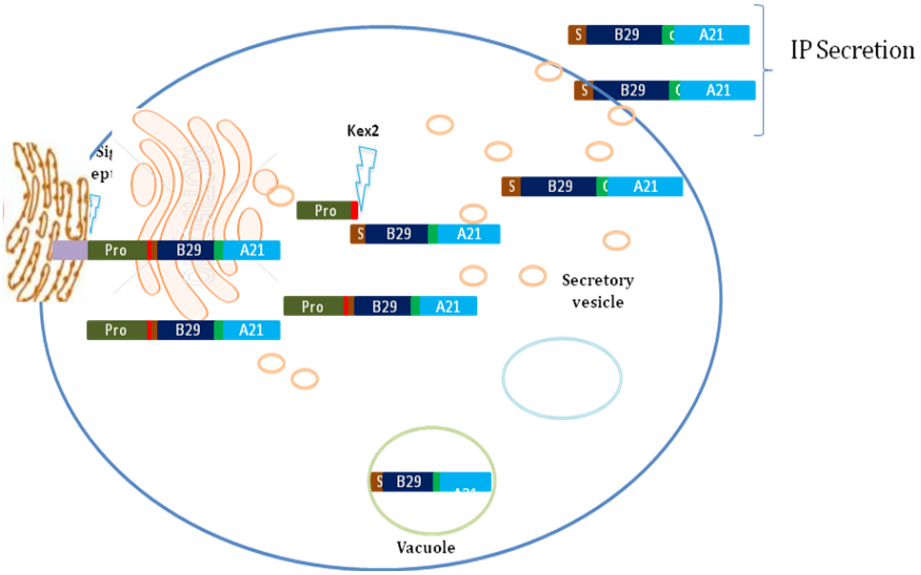

Fig 1.1: Secretion Pathway of Insulin precursor in *P. pastoris.*

1.1.3 Transpeptidation Reaction:

Transpeptidation is a kind of reaction involving the transfer of one or more amino acids from one peptide chain to another, as by transpeptidase action, or of a peptide chain itself, as in bacterial cell wall synthesis.[21]

1.2 What is Diabetes?

Diabetes is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively. Insulin is a hormone produced in the pancreas that allows glucose from food to enter the body's cells where it is converted into energy needed by muscles and tissues to function. A person with diabetes does not absorb glucose properly, and glucose remains circulating in the blood (a condition known as hyperglycaemia) damaging body tissues over time. This damage can lead to disabling and life-threatening health complications.[22]

There are three main types of diabetes:

• Type 1 diabetes

• Type 2 diabetes

• Gestational diabetes

1.2.1 Type 1 diabetes:

Type 1 diabetes is caused by an autoimmune reaction, where the body's defense system attacks the insulin-producing beta cells in the pancreas. As a result, the body can no longer produce the insulin it needs. Why this occurs is not fully understood. The disease can affect people of any age, but usually occurs in children or young adults. People with this form of diabetes need insulin every day in order to control the levels of glucose in their blood. Without insulin, a person with type 1 diabetes will die.Type 1 diabetes often develops suddenly and can produce symptoms such as:

• Abnormal thirst and a dry mouth
• Frequent urination
• Lack of energy, extreme tiredness
• Constant hunger
• Sudden weight loss
• Slow-healing wounds
• Recurrent infections
• Blurred vision

People with type 1 diabetes can lead a normal, healthy life through a combination of daily insulin therapy, close monitoring, a healthy diet, and regular physical exercise. The number of people who develop type 1 diabetes is increasing. The reasons for this are still unclear but may be due to changes in environmental risk factors, early events in the womb, diet early in life, or viral infections.[22]

1.2.2 Type 2 diabetes:

Type 2 diabetes is the most common type of diabetes. It usually occurs in adults, but is increasingly seen in children and adolescents. In type 2 diabetes, the body is able to produce insulin but either this is not sufficient or the body is unable to respond to its effects (also known as insulin resistance), leading to a build-up of glucose in the blood. Many people with type 2 diabetes remain unaware of their illness for a long time because symptoms may take years to appear or be recognized, during which time the body is being damaged by excess blood glucose. They are often diagnosed only when

complications of diabetes have already developed. Although the reasons for developing type 2 diabetes are still not known, there are several important risk factors. These include:

• Obesity
• Poor diet
• Physical inactivity
• Advancing age
• Family history of diabetes
• Ethnicity
• High blood glucose during pregnancy

1.2.3 Gestational diabetes:

Women who develop a resistance to insulin and subsequent high blood glucose during pregnancy are said to have gestational diabetes (also referred to as gestational diabetes mellitus or GDM). Gestational diabetes tends to occur around the 24th week of pregnancy. The condition arises because the action of insulin is blocked, probably by hormones produced by the placenta. As gestational diabetes normally develops later in pregnancy, the unborn baby is already well-formed but still growing. The immediate risk to the baby is therefore not as severe as for those whose mother had type 1 diabetes or type 2 diabetes before pregnancy (a condition known as diabetes in pregnancy). Nonetheless, uncontrolled gestational diabetes can have serious consequences for both the mother and her baby.[22, 23]

1.3 The Pancreas, Insulin, and Blood Glucose Regulation:

The pancreas is a unique organ because it serves both an exocrine and endocrine function. Composed primarily of acinar cells, it produces roughly 1200 to 1500 mL of pancreatic juice per day. Pancreatic juice is a high pH mixture that consists primarily of water, enzymes, and electrolytes.[24] Enzymes include trypsinogen, which is activated by enterokinase to form trypsin, carboxypeptitase, and chymotrypsin. The pancreas also produces amylases, lipases, and proteases. Islets of Langerhans, responsible for the endocrine function of the pancreas, are dispersed between the aciner cells. This structure contains beta cells that sense and secrete insulin.[24] Insulin is a hormone that causes most body cells to take up glucose from the blood and store it as glycogen in fat cells, muscles

or the liver. When insulinis low, and glycogen is not taken up in sufficient quantities, the body begins to 6 use fat as an energy source. Insulin is also a component of amino acid uptake as well as a number of important anabolic processes. The pancreas is stimulated to produce and release insulin when the autonomic nervous system senses food intake as well as when proteins are digested or glucose is found in the blood. Carbohydrates and sugared goods are the main sources of glucose in the body.[25] Glucose enters pancreatic β cells causing the release of insulin. Insulin decreases glucose production in the liver and stimulates glucose uptake, utilization and storage. Fat cells release free fatty acids that reduce glucose uptake in the cells and increase glucose production in the liver. The fat cell also secretes adipokines which regulate food intake, energy expenditure and insulin sensitivity.[26] Typically, insulin manufacture starts by creating preproinsulin in the ribosomes. The preproinsulin is converted to proinsulin, and is moved to thesecretory granules of the Golgi complex. When stimulated, in this case by acidification, proinsulin is folded upon itself and a central portion is cleaved with prohormone convertases PC1 and PC2. This result is C-peptide and the α and β chain of insulin. Finally, carboxypeptidase E creates mature insulin by causing the formation of disulfide bonds.[25, 27]

1.4 Insulin Secretion Pathway:

The most well understood and presumably the most important insulin release pathway is in response to blood glucose levels. Insulin is signaled to release from the vesicle with the entry of glucose into the β cell via GLUT27 facilitated diffusion. This receptor only exists in the pancreas, liver, kidneys and some neurons in the brain and is unique in that it does not require insulin in order to uptake glucose. Once inside the cell, the glucose molecule is phosphorolated and is no longer able to exit the cell. Glucose undergoes glycolysis to form ATP. ATP instigates the opening of the calcium channels, which causes the cell to depolarize. An increase in calcium concentration triggers the secretory granules of the β cells to release insulin into the bloodstream.[25] When released into the bloodstream, in order to produce a metabolic effect, insulin binds to the insulin receptor. Once this happens, GLUT4, the typical insulin-requiring receptor that allows glucose to enter the cells, moves into the plasma membrane and allows glucose to enter the cell. Once glucose goes into the cell, it is phosphorolated to prevent the molecule from leaving

the cell. Glucose goes through glycolysis to form ATP, which is stored inside as an energy source. Although all body cells must go through this process to produce ATP, this process has the highest throughput in liver and muscle cells.[25] This process is depicted in Fig:1.2. The lack of insulin results in a decrease of glucose utilization, which can lead to plasma glucose levels as high as 300-1200 mg/100 mL.[28] Type 2 diabetes is the term for patients who produce low levels of insulin, are insulin resistant or both. These patients may opt for insulin treatment when other medications to improve insulin production and diet fail. Insulin is also a part of a number of anaerobic cascades, such as the reduction of sex hormone binding globulin. Sex hormone binding globulin is a compound that binds to estradiol and testosterone rendering them inactive. Because of this fact, insulin resistance can lead to polycystic ovary syndrome in women. This is due to the fact that the extra insulin needed to control blood glucose levels also activates additional testosterone via its interaction with sex-hormone binding globulin.[29] One known drawback to insulin therapy is that there is no commercially available insulin formulation that includes C-peptide. As described earlier, C-peptide is also a derivative of proinsulin. C-peptide is utilized in repair of the muscular layer of the arteries. It also has been shown to reduce the occurrence of cardiovascular problems, as well as neuropathy in diabetic patients. One study showed that C-peptide may be capable of reversing neuropathy effects. In addition, it has been shown to have significant positive effects on glomerular function.[30]

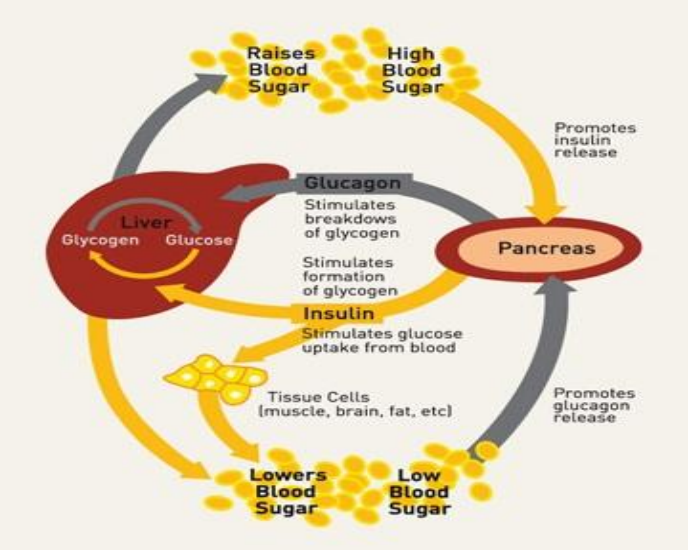

Fig. 1.2: Insulin Secretion Pathway.

1.5 Biosynthesis of Insulin from Proinsulin:

Insulin is synthesized in significant quantities only in beta cells in the pancreas. The insulin mRNA is translated as a single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin.Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide, thereby generating the mature form of insulin. Insulin and free C peptide are packaged in the Golgi into secretory granules which accumulate in the cytoplasm. When the beta cell is appropriately stimulated, insulin is secreted from the cell by exocytosis and diffuses into islet capillary blood. C peptide is also secreted into blood, but has no known biological activity.[31]

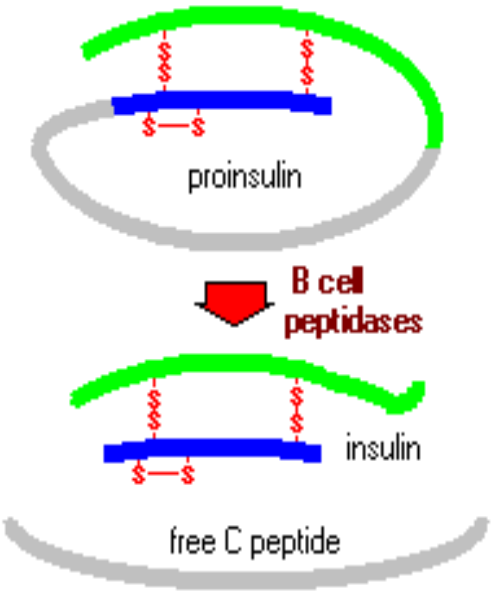

Fig.1.3: Biosynthesis of Insulin from Proinsulin.

1.6 Structure of insulin:

The human insulin is comprised of 51 amino acids and has a molecular weight of 5808 Da. The mature insulin, thus formed consists of an A-chain with 21 amino acids and a B-chain containing 30 amino acids and both polypeptides linked together by two disulphide bonds. Besides, the A-chain has an intrachain disulphide bond.[32]

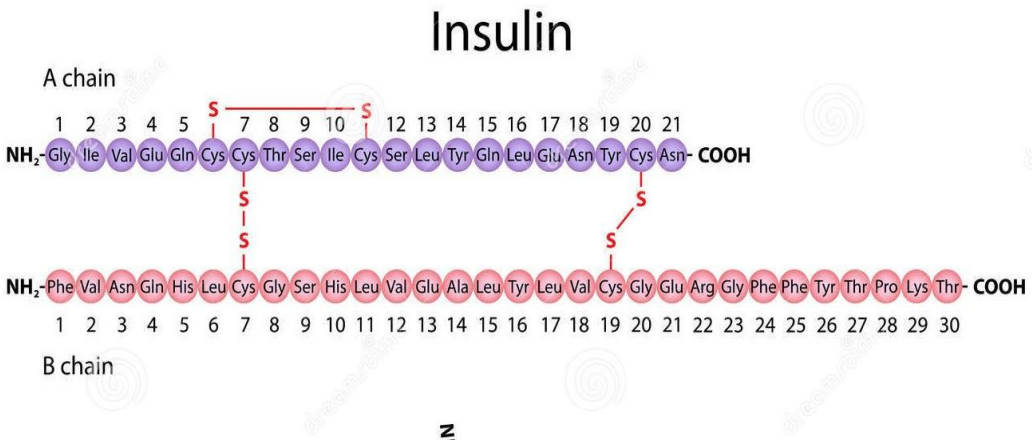

Fig.1.4: Structure of insulin.

1.7 Worldwide Diabetes status:

The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030. The prevalence of diabetes is higher in men than women, but there are more women with diabetes than men. The diabetes mellitus in urban population in developing countries is projected to double between 2000 and 2030.[33] About 1.1 million people were estimated to have died due to diabetes in 2005 and almost 80% of diabetes deaths occur in low- and middle-income countries and mostly people under the age of 70 years; 55% of diabetes deaths are in women.[34] The high incidence of tuberculosis (TB) in patients with diabetes mellitus (DM) has been a great concern for several years.[35] Diabetes Mellitus results in several medical, psychological and sexual dysfunction. Sexual dysfunction (SD) is known to be prevalent in both men and women with diabetes mellitus, but the sexual problems of women with diabetes mellitus have received much less attention in research and practice.[36]

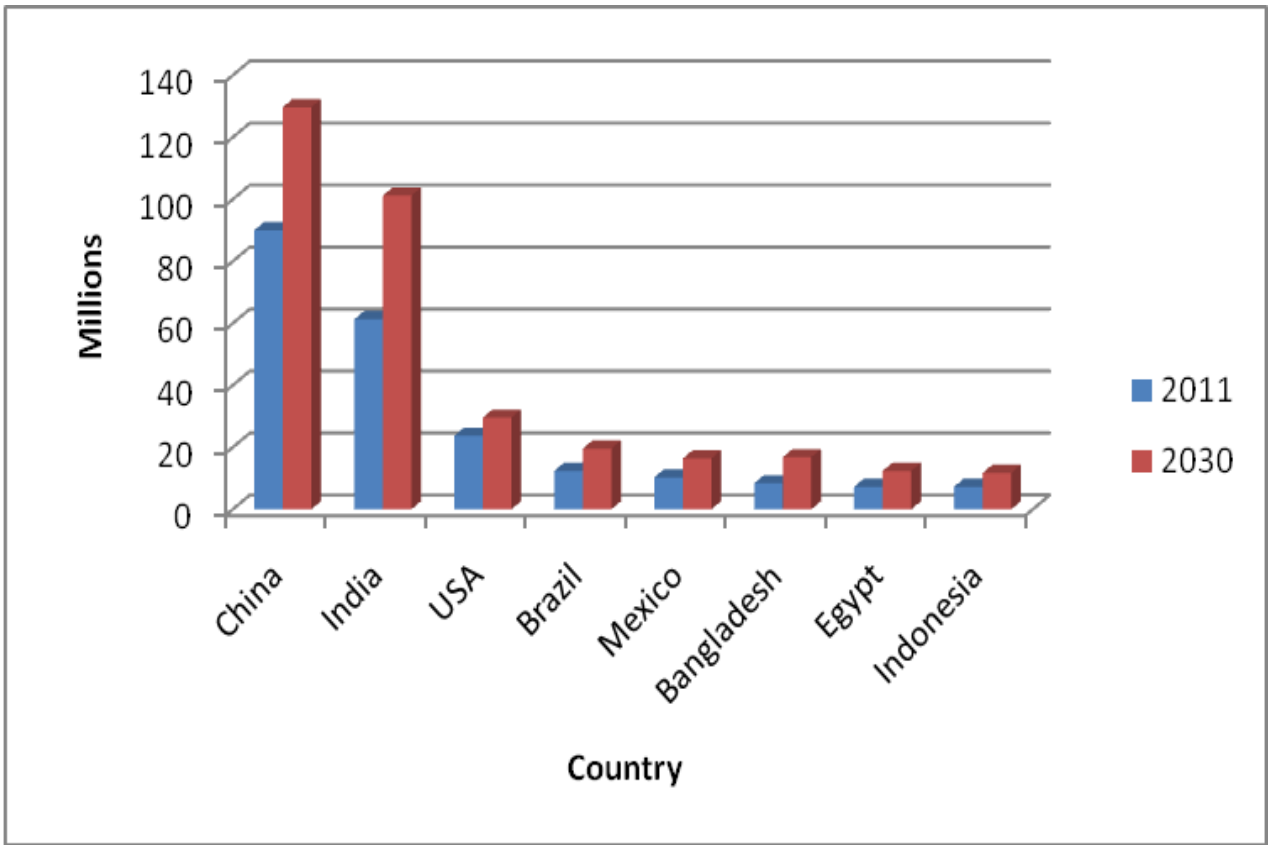

Fig 1.5: Projected Increase of Diabetes Patients worldwide from 2011 to 2030 (Diabetes Atlas).

1.8 Present Status in Bangladesh:

Diabetes mellitus possess a serious threat to developing countries like Bangladesh. According to latest WHO estimate about 7 million that means 70 lack people in Bangladesh have diabetes. The people of rural Bangladesh are rarely able to enjoy the benefit of the modern treatment progress of this disease.[37] The majority of the 382 million people with diabetes are aged between 40 and 59, and 80% of them live in low- and middle-income countries. All types of diabetes are on the increase, type 2 diabetes in particular: the number of people with diabetes will increase by 55% by 2035. An additional 21 million cases of high blood glucose in pregnancy are estimated to contribute to the global burden of diabetes. That is equivalent to 17% of live births to women in 2013 that had some form of high blood glucose in pregnancy.

In human as well as financial terms, the burden of diabetes is enormous, provoking 5.1 million deaths and taking up some USD 548 billion dollars in health spending (11% of the total spent worldwide) in 2013.[38]

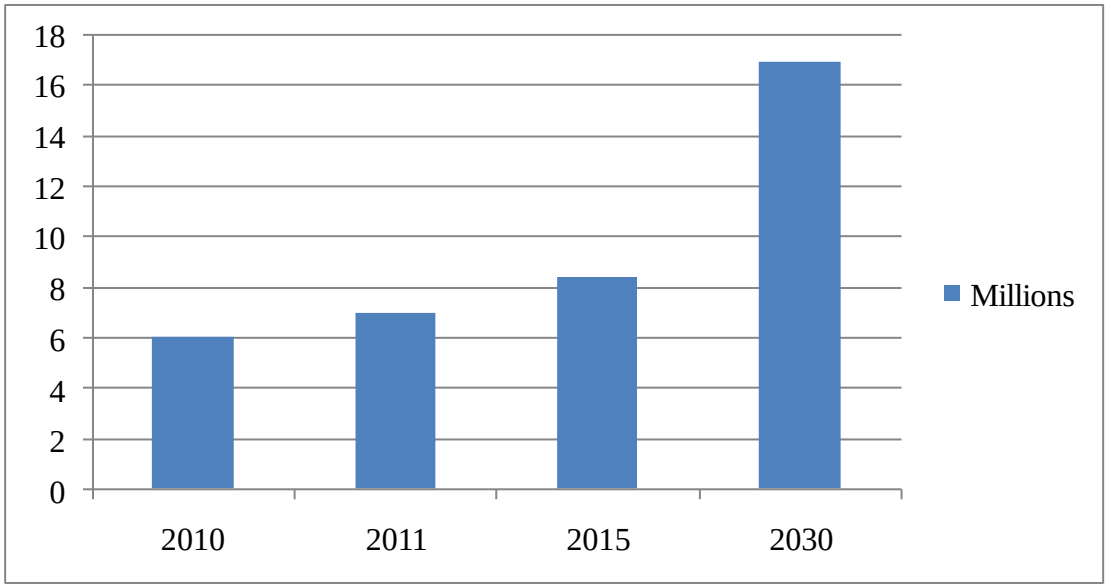

Fig1.6: Projected Increase of Diabetes Patients in Bangladesh (Diabetes Atlas).

1.9 Objective of the study:

Transpeptidation reaction is very critical for recovery of insulin at the same time the chemicals involve in this reaction trypsin and O-tert-Butyl-L-threonine –tert-butyl ester (O-Thr-ester) are highly expensive. The objective of the present study was to develop a protocol where certain parameters can be changed in congruence with published data to reduce the requirement for these two expensive chemicals for preparation of active insulin. The major focus was to use low amount of trypsin and O-Thr-ester for the transpeptidation reaction as well as changing the organic solvent composition, water content, pH, time and temperature. In this study, a two-step transpeptidation reaction has been proposed instead of one-step reaction process by separating the cleavage step from the coupling step so that each reaction can be performed under its optimal condition and the process is made simple, efficient, easy to scale-up and cost effective for the pharmaceutical production of human insulin.

Chapter 2

Materials and Methods

2.1 Materials:

P. pastoris expressing the recombinant human insulin precursor fusion protein (EEAEAEAEPK-B1–29-AAK-A1–21) was constructed and stored in the Incepta laboratory MRL. Tris hydroxymethyl aminomethane (Tris), H-Thr(tBu)-OtBu (O-Thr-ester), and trypsin were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile (HPLC grade) and TFA were purchased from J&K Chemical (Shanghai, China). CM Sepharose FF and SOURCE 30 PRC were purchased from GE Healthcare (Fairfield, CT, USA). Other reagents were of analytical grade. HPLC (Shimadzu, Japan) was used chromatographic analysis. The data were collected and calculated using Labsolution system (Shimadzu, Japan).

2.2 Methods:

2.2.1 One-step transpeptidation of insulin precursor fusion protein

The insulin precursor fusion protein 50mg/mL was taken and Dimethylformamide (DMF) was added to get a 75% organic ratio. 7mM Calcium chloride $CaCl_2$ solution was added to the solution and mixed vigorously. Then O-Thr-ester was added at the molar ratio of 35:1with insulin precursor. Tris buffer was added to adjust the (pH 7.00) and the final concentration of insulin precursor fusion protein was 7.10 mM. Trypsin was finally added at a ratio of trypsin–insulin precursor fusion protein to 1:50 (w/w). The reaction system was stirred at 250 rpm at room temperature.. The transpeptidation samples were obtained at 0 H (sampling immediately after all the reactants were mixed), 6^{th}, 10^{th}, 14^{th}, 18^{th}, 22^{nd}, 24^{th} and 40^{th} hour, respectively. The sample solution was immediately diluted for (15-20) times with 100 mM acetic acid to stop the reaction. The conversion ratios of the insulin precursor fusion protein to insulin ester at different reaction time points were then analyzed using high-performance liquid chromatography (HPLC).

2.2.2 Two-step transpeptidation of insulin precursor fusion protein

Insulin precursor 50mg/ml present in 50% organic solvent was taken and tris was added to adjust the pH 8.0.Then trypsin was added to obtained insulin precursor: trypsin ratio 1:50(w/w) and digestion was performed in different temperatures to get better cleavage. Sample was taken at 0.45hr, 1.0hr & 1.15 hr.

After 1hr of trypsin digesion DMF was added to the sample to get 75% organic ratio. Then O-Thr-ester was added in 1:35 molar ratio considering the total volume, finally pH was adjusted to 6.0 and 7.0 respectively by adding Tris and 5N HCl. The conversion ratios of the insulin precursor fusion protein to insulin ester at different reaction time points and pH were analyzed

using high-performance liquid chromatography (HPLC) and shown as the peak area of insulin esters in the HPLC chromatogram (peak area of insulin precursor fusion protein + peak area of the cleavage intermediates + insulin ester peak area).

100 mM acetic acid was used to terminate the reaction. The conversion ratio of desB30 to insulin ester at different reaction time and temperature was then analyzed using HPLC and shown as the peak area of insulin esters in the HPLC chromatogram (peak area of desB30 + insulin ester peak area).

Chapter 3

Results

3.1 One-Step Transpeptidation Reaction:

3.1.1 Effect of trypsin:

One step transpeptidation reaction was performed with 3 (three) different trypsin : Insulinprecursor ratio(w/w) where the O-Thr-ester concentration remained constant . Among those reactions we found the best result i. 1:50(w/w) trypsin: IP where the O-Thr-ester :IPmolar ratio was 50:1(mol/mol).

Table 3.1: Conversion(%) of insulin precursor to insulin ester in three different Trypsin : IP ratio (w/w)

O-Thr-ester: IP (mol/mol)	**Trypsin : IP ratio (w/w)**		
	1:25	1:50	1:75
50:1	38	48	41

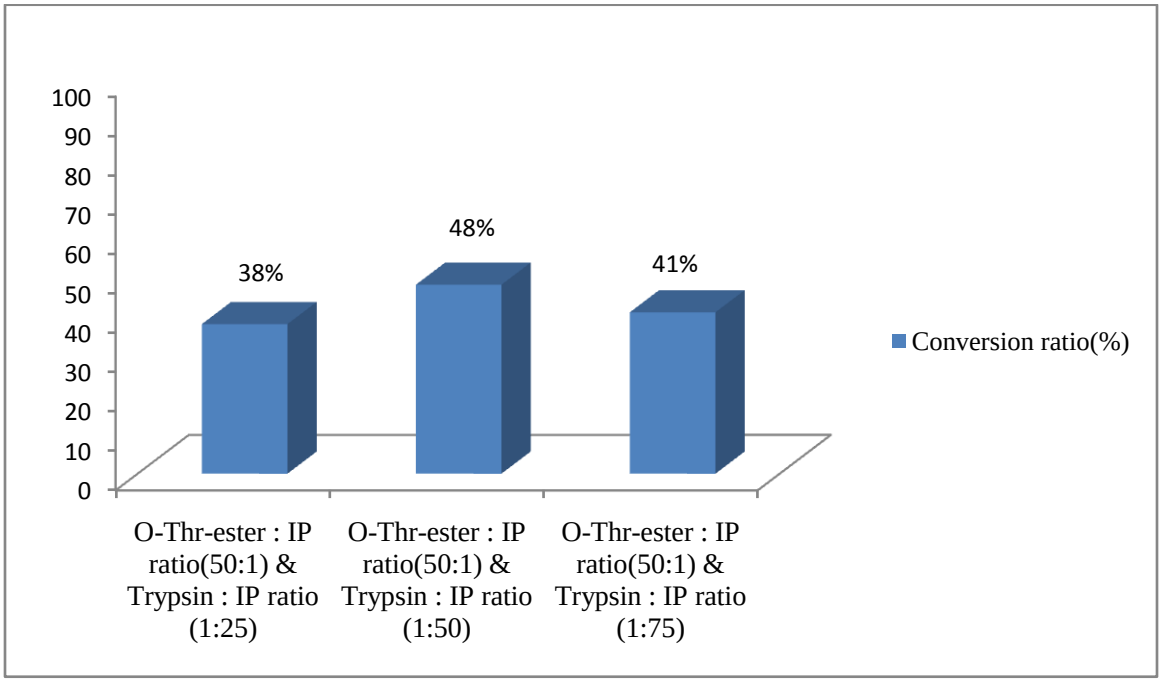

Fig 3.1: Conversion ratio(%) in three different Trypsin concentrations

3.1.2 Effect of O-Thr-ester:

To optimize O-Thr-ester consumption for one step transpeptidationreaction3 (three) different molar ratio of O-Thr-ester : IP were applied, where the trypsin concentration was remained

constant 1:50 (w/w) . In this reaction we found the best result with O-Thr-ester : IP molar ratio 35:1 and the trypsin: IP ratio 1:50(w/w)

Table 3.2: Conversion (%) of insulin precursor to insulin ester in three different molar ratios of O-Thr-ester: IP

Trypsin : IP ratio (w/w)	**O-Thr-ester : IPratio (mol/mol)**		
	50:1	35:1	25:1
1:50	46	47	19

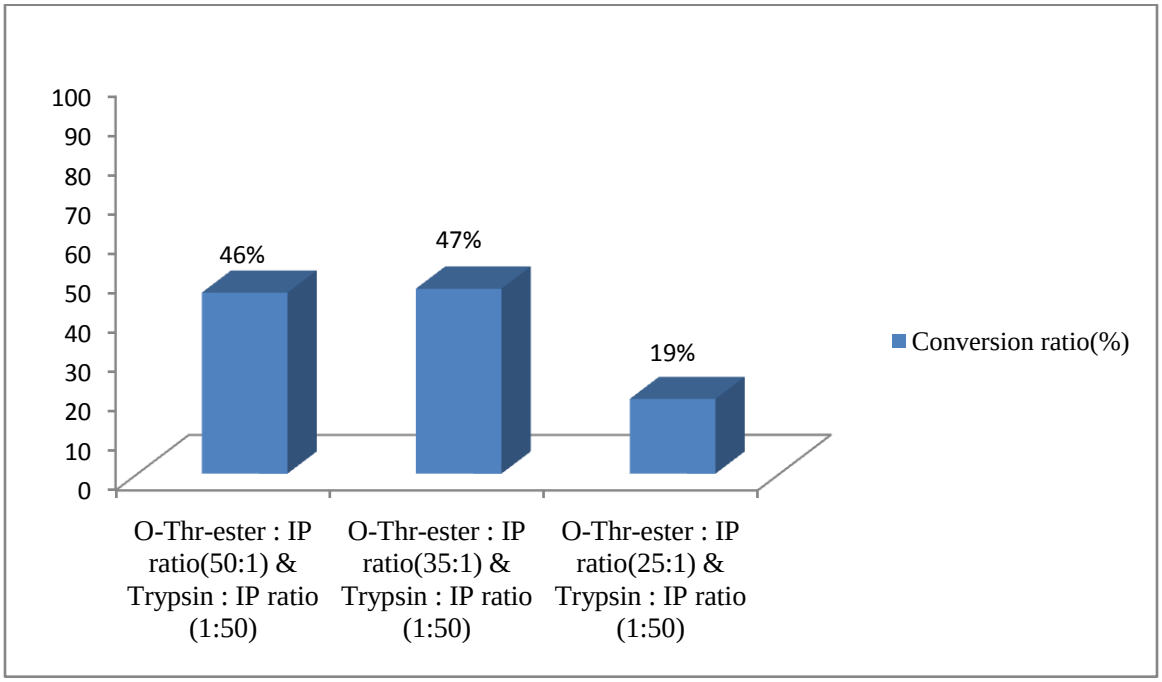

Fig 3.2: Conversion ratio(%) in three different O-Thr-esterconcentrations.

3.1.3 After fixing the O-Thr-ester and trypsine ratio reaction time was optimized to get the best yield in one-step transpeptidationreaction at 25^0C temperature. The reaction was carried out up to 40 hours and sampling were done at 6^{th}, 10^{th}, 14^{th}, 18^{th}, 22^{nd}, 24^{th} and 40^{th} hour.

Table 3.3: Conversion ratio (%) towards insulin ester at different time with same concentrations of O-Thr-ester& trypsin at 25^0Ctemperatures.

Reaction Hour	Trypsin:IP	O-Thr-ester :IP	pH	% of Un-reacted IP (P1)	% of cleavage but not ligated IP (P2)	% of total conversion of Insulin ester (P2)
6	1:50	35:1	7.0	45	15	33
10				38	16	36
14				36	14	37
18				33	15	39
22				30	14	40
24				28	16	47
40				20	19	40

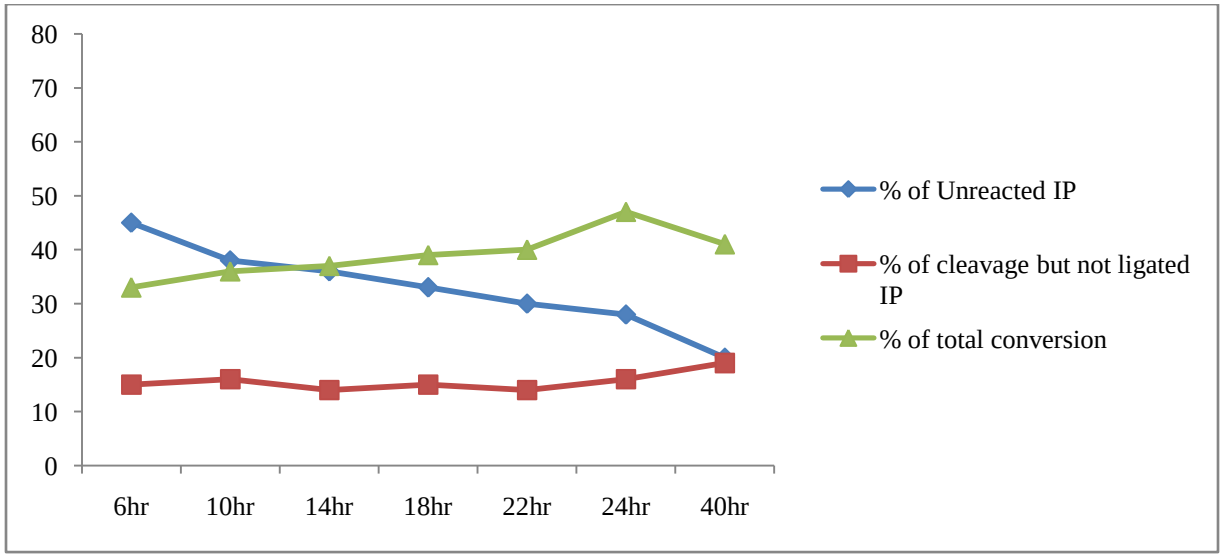

Fig 3.3: Conversion ratio (%) towards Insulin ester at different time.

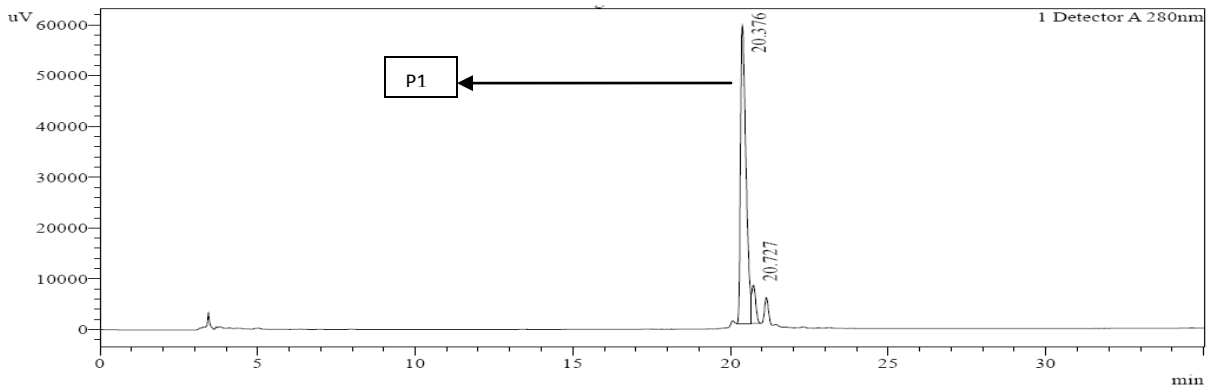

Fig3.4: RPC chromatogram of Insulin Precursor P1 is the main peak where the IP concentration was 50mg/mL.

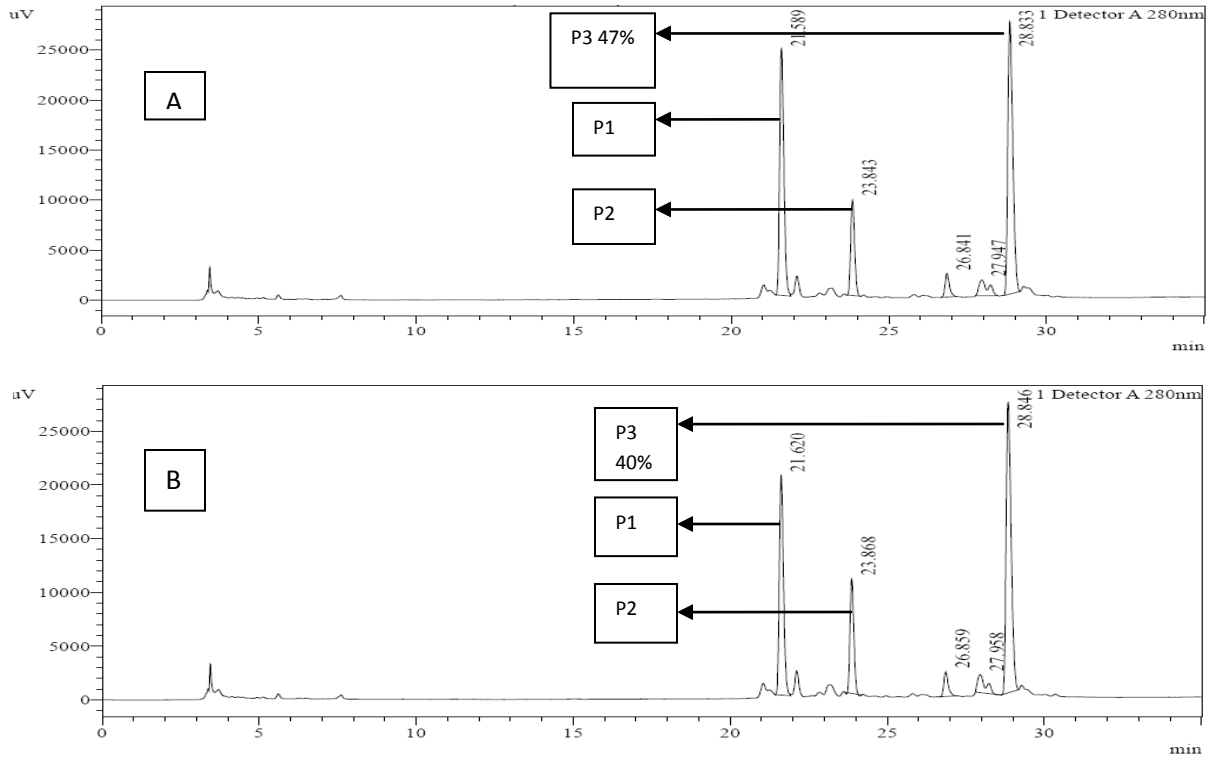

Fig3.5: HPLC chromatogram of One-step Transpeptidation reaction of insulin precursor fusion protein to insulin ester at different reaction times point 24 hr (A) and 40 hr (B). The transpeptidation reaction samples were taken and diluted 10 times with GAA. The elution was performed at 25 ^{0}C with a linear gradient bymobile phase A (0.1%, v/v, TFA in Milli-Q water) and mobile phase B (0.1%, v/v, TFA in acetonitrile) in a proportion of 20-46% B (0-38Min). The flow rate was 1.0 mL/min and column effluent was monitored at 280 nm. In chromatograms,P1

is insulin precursor fusion protein peak, P2 is insulin precursor desB30 peak and P3 is insulin ester peak.

3.2 Two step transpeptidation reaction:

In two-step transpeptidation reaction the total reaction was carried out into two individual stages.In step-1 trypsin digestion was performed with optimum reaction condition to get the height cleavage of IP. Then O-Thr-esterligation was done in step-2.

3.2.1 Step-1,enzymatic digestion:

In step-1, trypsin digestion was performed at room temperature and 35^0C temperature where the trypsin: IP ratio was 1:50(w/w) and the pH was 8.0. Reaction sample was taken at 0.45hr,1.0hr & 1.15 hr.At 35^0C temperature initial digestion rate was high compared to rate at room temperature but after 1hr reaction went reverse.

Table 3.4: Step-1, enzymatic digestion at room temperature(25^0C)

Reaction time in Hour	Trypsin : IP	pH	% of Un-reacted IP	% of insulin desB30
0.45 hr			15	81
1.0 hr	1:50	8.0	10	86
1.15 hr			17	81

Table 3.5: Step-1, enzymatic digestion at 35^0Ctemperature.

Reaction time in Hour	Trypsin : IP	pH	% of Un-reacted IP	% of insulin desB30
0.45 hr			12	84
1.0 hr	1:50	8.0	14	85
1.15 hr			17	76

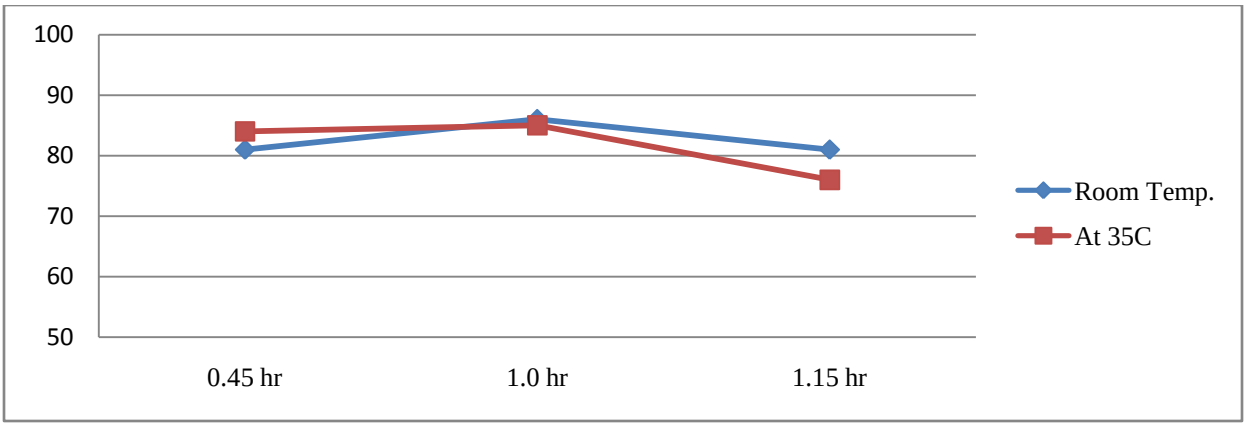

Fig 3.6: Step-1,enzymatic digestion at different temperature and conversion ratio (%) towards insulin desB30.

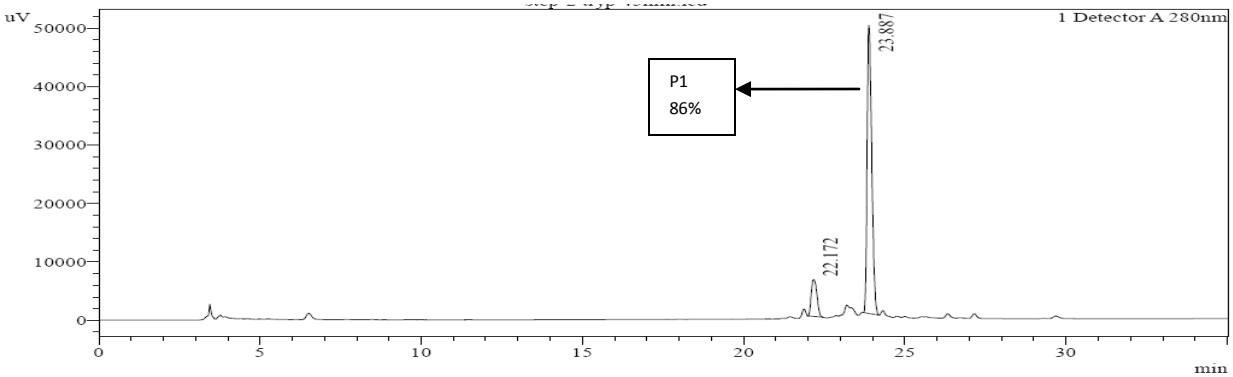

Fig 3.7:HPLC chromatogram of desB30 (P1), at room temperature after 1hr. The operation conditions were same as described in Fig.3.5.

3.2.2 Step-2,O-Thr-esterligationat Room Temperature:

In step-2,O-Thr-ester ligation was performed with digested IP (desB30) at pH 6.0 where the DMF was used to make 75% organic ratio in order to facilitate the reaction condition.

Table 3.6: step-2, O-Thr-ester ligation at room temperature(25^0C):

Reaction time in hour	O-Thr-ester : IP	% of Organic solvent (DMF)	pH	% of Un-reacted IP	% of insulin desB30	% of insulin ester
4 hr	35:1	75 %	6.0	35	59	4
6 hr				33	58	4
8 hr				30	55	10
10 hr				28	52	13

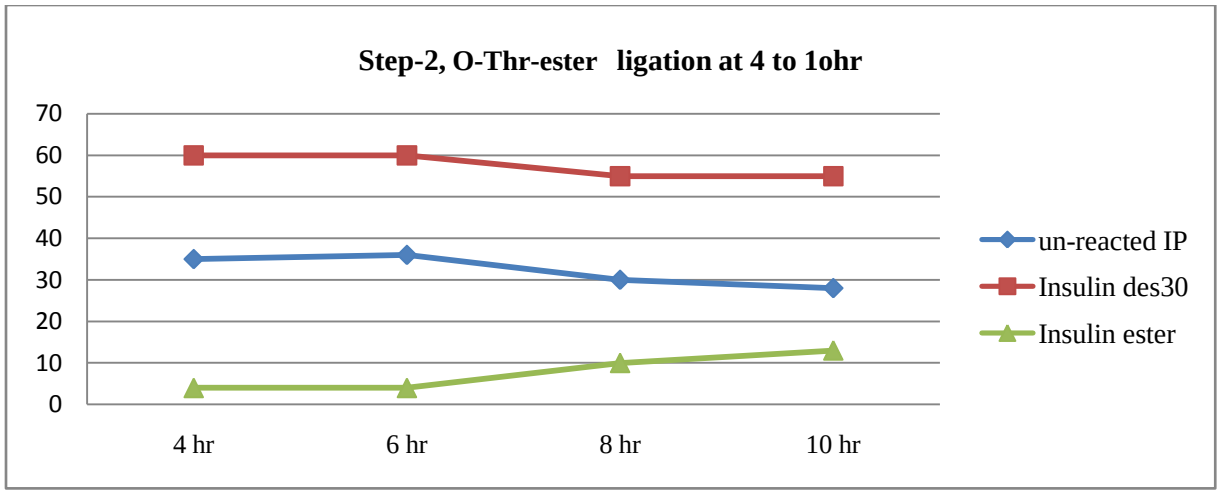

Fig 3.8: Conversion ratio (%) ofdes-B30 to Insulin ester at different time.

3.2.3 Step-2,O-Thr-esterligation at 12^0C Temperature:

As the result obtained from room temperature was not satisfactory, so the step-2 O-Thr-ester ligation was also performed at 12^0C with digested IP to observe the temperature effect on the reaction at pH 6.0 where the DMF was used as organic solvent.

Table 3.7: Step-2,O-Thr-ester ligation at 12^0C temperature

Reaction time in Hour	O-Thr-ester : IP	% of Organic solvent (DMF)	pH	% of Un-reacted IP	% of insulin desB30	% of insulin ester
4 hr	35:1	75%	6.0	35	60	4
6 hr				36	60	4
8 hr				30	55	10
10 hr				28	55	18

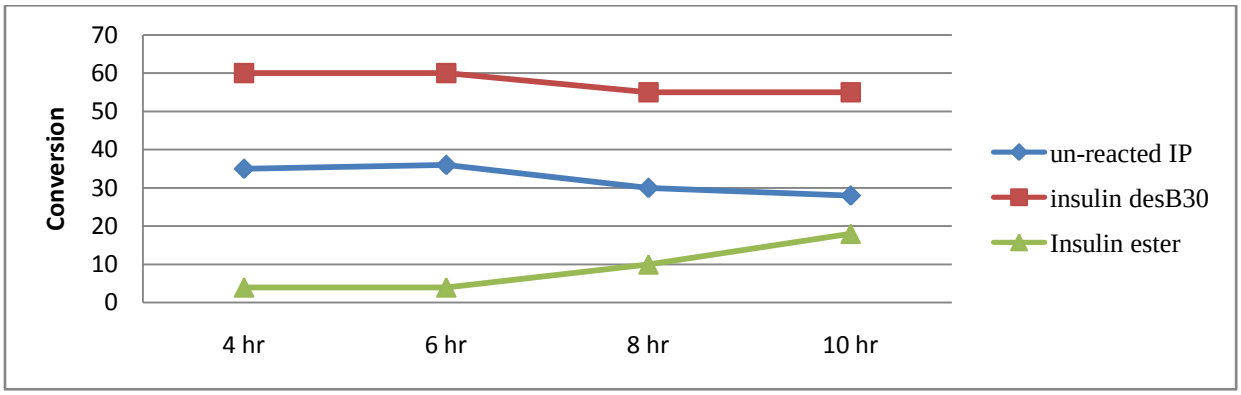

Fig 3.9: Conversion ratio (%) of des-B30 toInsulin ester at 12^0C temperature.

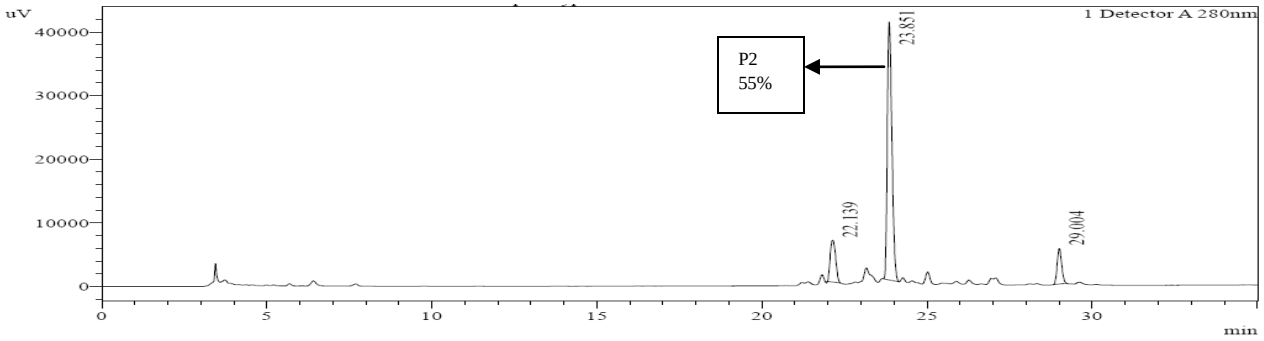

Fig 3.10:HPLC chromatogram for (%) of insulin precursor (p1), des-B30 (p2) &Insulin ester(p3) at 12^0C temperature and 10hr.

P3
18%

3.2.4 Step-2,O-Thr-esterligation at pH 7.0:

As the satisfactory result was not found at pH 6.0, sopH of the reaction was changed from 6.0 to 7.0 and rest of the reaction parameters kept unchanged to observe the effect of pH in O-Thr-ester ligation process. This reaction also performed at room temperature and 12^0C temperature.

Table 3.8: Step-2,,O-Thr-ester ligation at 25^0C temperature

Reaction time in hour	O-Thr-ester : IP	% of Organic solvent (DMF)	pH	% of Un-reacted IP	% of insulin desB30	% of insulin ester
4 hr	35:1	75 %	7.0	29	56	6
6 hr				25	51	12
8 hr				23	49	19
10 hr				19	44	24

Table 3.9: Step-2,O-Thr-ester ligation at 12^0C temperature

Reaction time in hour	O-Thr-ester : IP	% of Organic solvent (DMF)	pH	% of Un-reacted IP	% of insulin desB30	% of insulin ester
4 hr	35:1	75%	7.0	31	38	5
6 hr				30	41	9
8 hr				28	49	13
10 hr				27	38	18

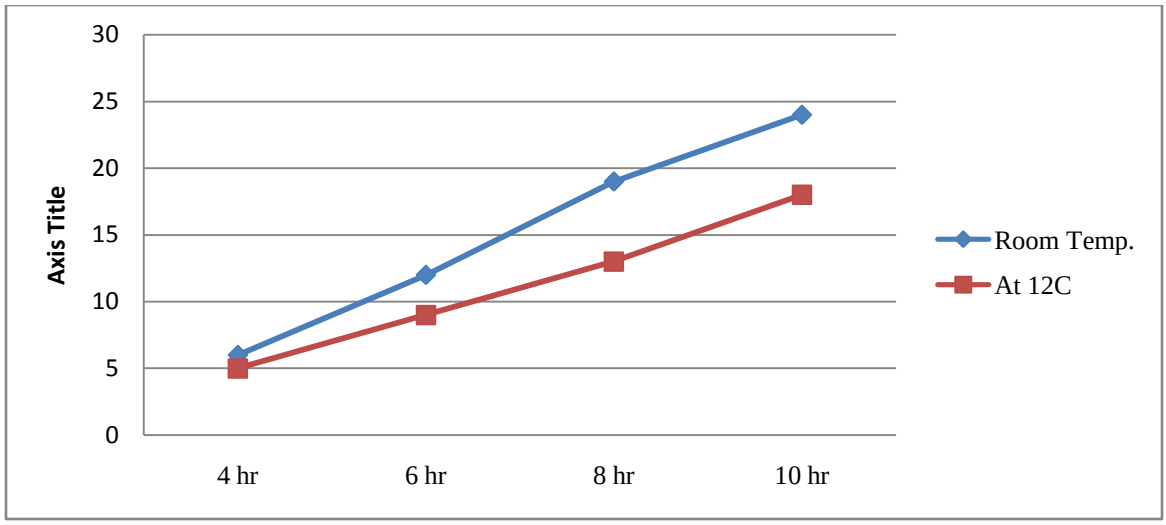

Fig 3.11: Step-2,O-Thr-ester ligation at different temperature in pH 7.0

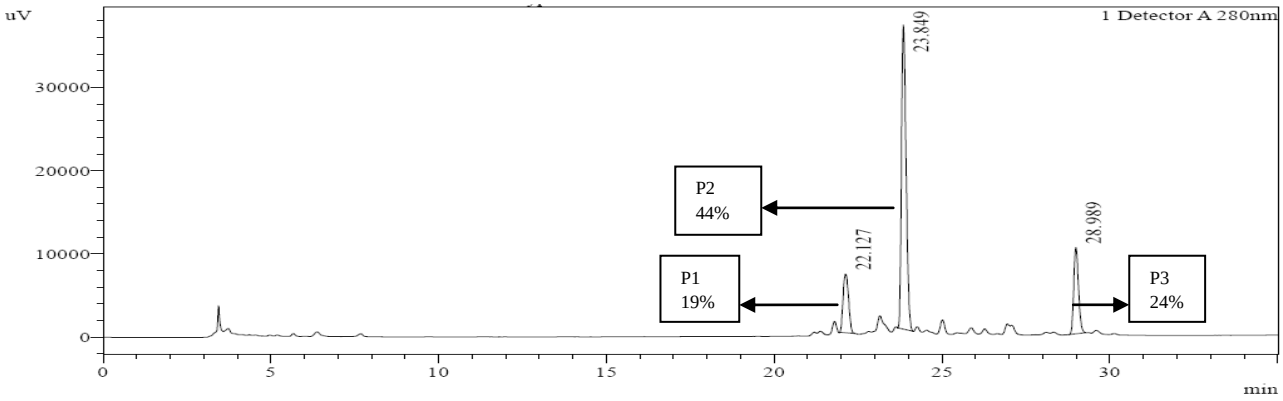

Fig 3.12:HPLC chromatogram for (%) of insulin precursor (p1), des-B30 (p2) &Insulin ester(p3) at 25^0C temperature and 10hr.

3.2.5 Two-step transpeptidation reaction with EtOH:

Maximum coupling of insulin ester was found in DMF as an organic solvent at25^0C&pH was 7.0 and the yield was 24% Fig: 3.13. To increase the yield in ligation stage and observe the effect of different organic solvent, DMF was replaced by Ethanol and the reaction was performed at room temperature 25^0Cand 12^0C.

Table 3.10: Step-2,O-Thr-ester ligation at 12^0C temperature

Reaction time in hour	O-Thr-ester : IP	% of Organic solvent (EtOH)	pH	% of insulin desB30	% of insulin ester
4 hr	35:1	75%	7.0	78	19
6 hr				47	44
8 hr				36	52
10 hr				28	64

Table 3.11: Step-2,O-Thr-ester ligation at 25^0C temperature

Reaction time in hour	O-Thr-ester : IP	% of Organic solvent (EtOH)	pH	% of insulin desB30	% of insulin ester
4 hr	35:1	75%	7.0	73	20
6 hr				39	48
8 hr				12	74
10 hr				11	69

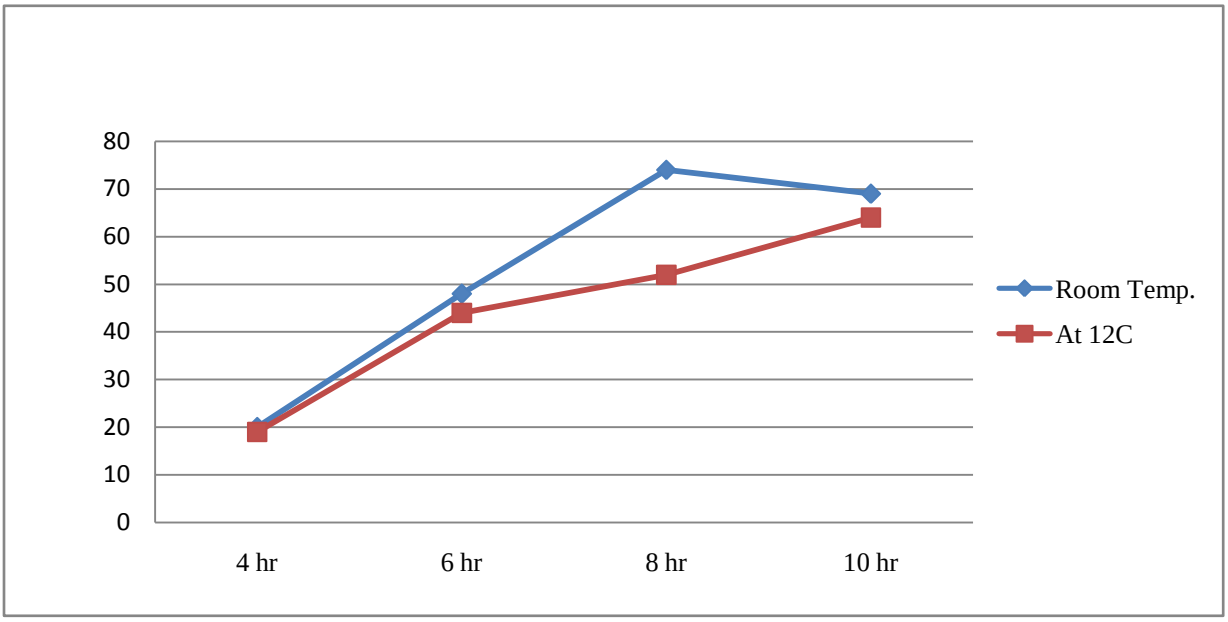

Fig 3.13: Step-2, O-Thr-ester ligation at different temperature in pH 7.0 with EtOH.

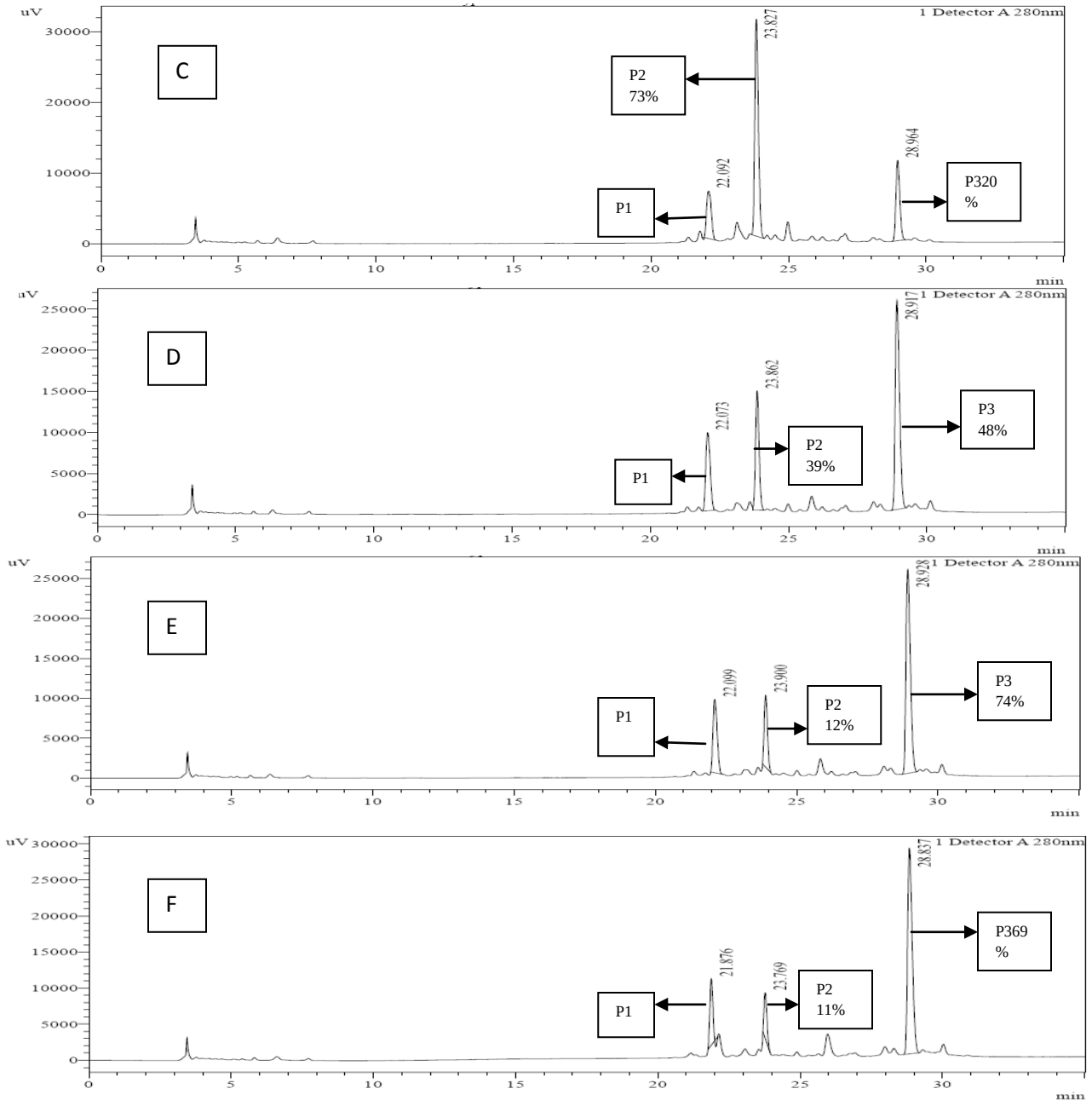

Fig3.14: HPLC chromatogram of Two-step Transpeptidation of insulin precursor fusion protein to insulin ester at different reaction time point 04hr, 06hr, 08hr and 10hr (C to F). The transpeptidationreaction samples were taken and diluted 10 times with GAA. The elution was performed at 25 C with a linear gradient by mobile phase A (0.1%, v/v, TFA in Milli-Q water) and mobile phase B (0.1%, v/v, TFA in acetonitrile) in a proportion of 20-80% B (0-30Min). The flow rate was 1.0 mL/min and column effluent was monitored at 280 nm. In chromatograms P1 is insulin precursor fusion protein of which peakretention time (RT)is around 21 min, P2 is

single-chain insulin precursor, desB30 peak RT is around 23 min and P3 is insulin ester peak RT around 28 min.

3.2.6 Comparison between one-step and two-step reaction:

Through the two-step transpeptidation method, the total the conversion of insulin ester increased 57.44 % and the reaction time was reduced 58.33% by using the same amount of trypsin and O-Thr-ester compared with the one-step method.

Table 3.12: Comparison between one-step and two-step reactionat 25^0C temperature

Reaction method	Reaction time hr	Temp.	Trypsin:IP (w/w)	O-Thr-ester : IP (mol/mol)	% of conversion
One-step transpeptidation	24	25	1:50	35:1	47
Two-step transpeptidation	10	25			74

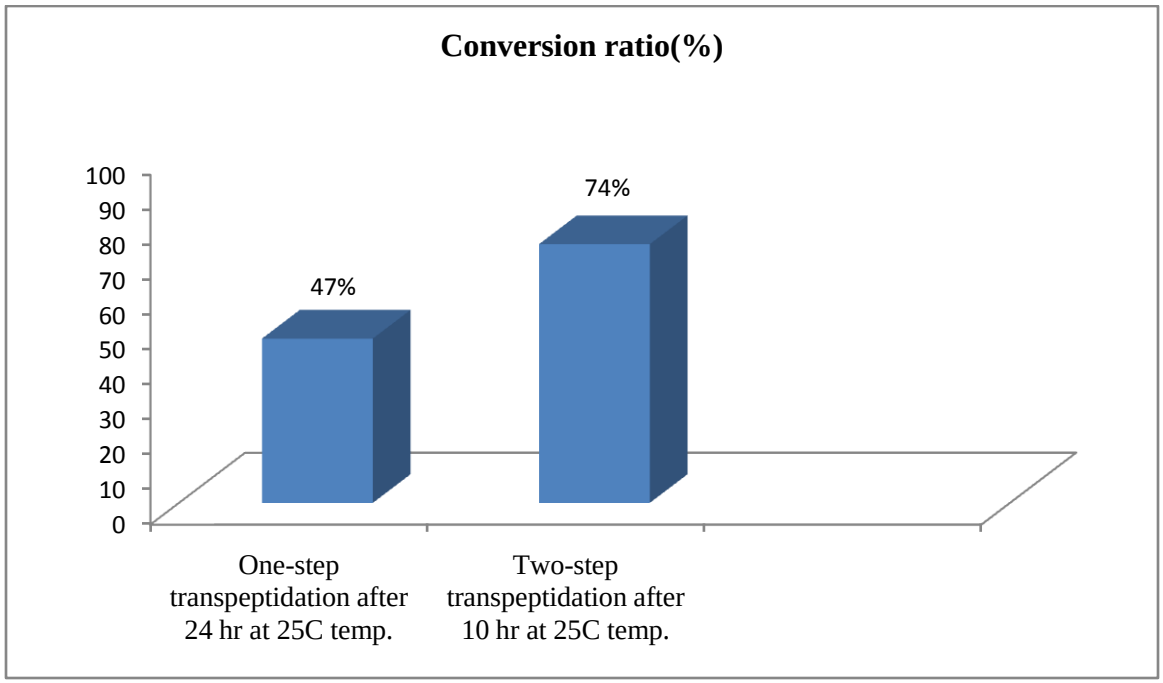

Fig 3.15: Comparison between one-step and two step reactions at different reaction hrs.

Chapter 4

Discussion

4.1 One-step transpeptidation reaction of insulin precursor fusion protein:

To obtain a high conversion ratio of the one-step transpeptidation reaction, a high-substrate concentration was required, and the molecular ratio of o-tert-Butyl-L-threonine tert-butyl ester (O-Thr-ester) to substrate was as high as 100:1.[12] It is reported that low-water content in the conversion reaction mixture was critical in case of achieving the high conversion ratio of the transpeptidation reaction. The selection of an appropriate organic solvent, such as ethanol, DMF, or (*N*,*N*)-dimethyl acetamide is very important, and the pH value should be neutral or weakly acidic.[38-40] The high concentrations of organic solvent usually inhibit or reduce trypsin activity. So a large amount of trypsin is necessary to sustain and efficiently catalyze the reaction. In the one-step transpeptidation reaction, the mass ratio of trypsin and insulin precursor should not be lower than 1:5.[41]

The insulin precursor fusion protein expressed in *P. pastoris* is a single-chain peptide fused with a spacer peptide localized at its N-terminus and containing three trypsin cleavage sites in the polypeptide chain. As a single-chain peptide, if the insulin precursor fusion protein is digested into desB30, then it is possible to convert it to insulin ester. Here, insulin precursor fusion protein is digested at three cleavage site by trypsin leave the possibilities to form a single-chain insulin precursor (removing spacer peptide), double-chain insulin precursor (cleaving behind the connecting peptide AAK), and desB30 (des-threonine^{B30} human insulin).[13]

In our experiment insulin precursor fusion protein samples are analyzed with different trypsin-IP & O-Thr-ester ratio, at different reaction times in one-step transpeptidation reaction using HPLC. In the HPLC profiles of different reaction time (Figs. 3.5A&B), the insulin precursor fusion protein (P1 peak) was first digested to form single-chain insulin precursor (P2 peak), which subsequently formed desB30 after two cleavages, and finally formed insulin ester (P3 peak). In such conditions in one-step transpeptidation, the total reaction rate was very slow. When the reaction was performed for 6hr, only a small amount of insulin ester was formed. After reacting for 14hr, less than 40% of the insulin precursor fusion protein was converted into insulin ester. At 24 hr, the transpeptidation ratio increased to 47%.

However, when the reaction was performed for 40hr, the amount of by-products increased and the transpeptidation ratio decreased back to 40%. Moreover, the longer the reaction time, the more by-products were formed. After 40hr, the amount of by-products increased significantly

compared to that observed after 24 hr reaction.

In the one-step transpeptidation reaction solution, high concentrations of trypsin would inevitably result in an autolysis reaction of trypsin. These autolysis products were likely to participate in the transpeptidation reaction, and the constitution of by-products became very complex.[42] This increased the cost of downstream processes and decreased the yield of product after purification.

4.2 Two-step transpeptidation reaction of insulin precursor fusion protein:

Two-step transpeptidation reaction actually consisted of two successive reactions to convert insulin precursor fusion protein into human insulin ester.[43] The first reaction is the cleavage of insulin precursor fusion protein using trypsin to desB30, and the second reaction is an enzymatic-catalyzed coupling of O-Thr-ester to the terminal lysine^{B29} residue of desB30 to form human insulin ester in the presence of trypsin and O-Thr-ester. Trypsin cleavage preferentially occurs in an aqueous or low-concentration organic solvent. To shift the reaction toward synthesis in solution, both high concentrations of one reactant and high concentrations of the water–miscible organic cosolvent have been used to increase the yield of the target product. The coupling reaction was preferably performed at minimal water conditions, which reversed the enzymatic activity of trypsin from hydrolysis toward synthesis to generate human insulin ester. Because this coupling reaction could hardly happen under the optimal conditions of cleavage reaction [13], the reaction conditions of one-step transpeptidation was accumulated. However, the final product insulin ester increased slowly in the one-step reaction system, thus the cleavage process of the insulin precursor fusion protein was the rate-limiting step.[44]

To improve the conversion ratio, one-step transpeptidation was used to divide the protocol into two individual steps such that both the reactions could be performed under respective optimal conditions. In step-1, the insulin precursor fusion protein was cleaved by trypsin in low-concentration of organic solvent 50% EtOH which resulting conversion of 86% desB30 shown in (Fig:3.7). In step-2, O-Thr-ester was coupled to the terminal lysineB^{29} residue of desB30 to form insulin ester where, high-concentration organic solvent (75%) ratio was applied to get the maximum conversion. So maintain organic solvent ratio by only EtOH showed the better conversion (74%) than using 2:1 EtOH : DMF ration.

4.3 Comparison of one-step and two-step transpeptidation reaction:

i. The total conversion ratio of the two-step transpeptidation was approximately 74%, where the conversion ratio of the one-step transpeptidation was only 47%.

ii. Using the same amount of amount of trypsin and O-Thr-ester in two-step transpeptidation yield was improved up-to 57.44% as a result trypsin and O-Thr ester consumption minimized, compared with the one-step transpeptidation.

iii. The rate of desB30 coupling was very fast, such that the highest coupling ratio could be achieved within 10hr, a time point where there was nearly less by-product, which made the subsequent separation and purification relatively simple.

iv. Longer exposure time (24hr) for one step reaction that is extensively related to highest conversion ratio, also generate by-products results in increased difficulties in subsequent purification steps.

v. Henceforth, Compared with the one-step method, preparation of recombinant human insulin using two-step transpeptidation showed a fast reaction rate, low production cost, and high transpeptidation ratio.

Chapter 5

Conclusion

&

Recommendation

5.1 Conclusion:

Diabetes mellitus possess a serious threat to developing countries like Bangladesh both socially and financially. So, a low cost process needs to develop for the production of active human insulin. In this study, through two-step transpeptidation protocol we achieved an efficient, high-yield, low-cost process for the conversion of insulin precursor fusion protein to insulin ester which is an important stage for the production of active human insulin.

5.2 Recommendation:

i. Amount of trypsin and O-Thr-ester can be further adjusted to achieve more than 74% yield.
ii. Reaction time is an important factor to avoiding by-products so extensive sampling can be done during scale.

Chapter 6

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