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Recombinant DNA Technology in the Synthesis of Human Insulin

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ABSTRACT

Insulin is a hormone that regulates the amount of glucose (sugar) in the blood and is required for the body to function normally. Insulin is produced by cells in the pancreas, called the islets of Langerhans. These cells continuously release a small amount of insulin into the body, but they release surges of the hormone in response to a rise in the blood glucose level.

People who do not produce the necessary amount of insulin have diabetes. There are two general types of diabetes. The most severe type, known as Type I or juvenile-onset diabetes, is when the body does not produce any insulin. Type I diabetics usually inject themselves with different types of insulin three to four times daily. Dosage is taken based on the person's blood glucose reading, taken from a glucose meter. Type II diabetics produce some insulin, but it is either not enough or their cells do not respond normally to insulin. This usually occurs in obese or middle aged and older people. Type II diabetics do not necessarily need to take insulin, but they may inject insulin once or twice a day.

If the body does not produce any or enough insulin, people need to take a manufactured version of it. The major use of producing insulin is for diabetics who do not make enough or any insulin naturally. Human insulin is grown in the lab inside common bacteria. *Escherichia coli* is by far the most widely used type of bacterium, but yeast is also used. Researchers need the human protein that produces insulin. Manufacturers get this through an amino-acid sequencing machine that synthesizes the DNA. Manufacturers know the exact order of insulin's amino acids.

1)INTRODUCTION

Insulin is a hormone that is important for metabolism and utilization of energy from the ingested nutrients - especially glucose. Insulin is a protein chain or peptide hormone. There are 51 amino acids in an insulin molecule. It has a molecular weight of 5808 Da.Insulin is produced in the islets of Langerhans in the pancreas. The name insulin comes from the Latin "insula" for "island" from the cells that produce the hormone in the pancreas.

Insulin's structure varies slightly between species of animal. Both porcine (from pigs) and bovine (from cows) insulin are similar

to human insulin but porcine insulin resembles human insulin more closely.

What does insulin do?

Insulin has several broad actions including:

It causes the cells in the liver, muscle, and fat tissue to take up glucose from blood and convert it to glycogen that can be stored in the liver and muscles

Insulin also prevents the utilization of fat as an energy source. In absence of insulin or in conditions where insulin is low glucose is not taken up by body cells, and the body begins to use fat as an energy source

Insulin also controls other body systems and regulates the amino acid uptake by body cells

It has several other anabolic effects throughout the body as well.

Insulin is synthesized in significant quantities only in beta cells in the pancreas. It is secreted primarily in response to elevated blood concentrations of glucose. Insulin thus can regulate blood glucose and the body senses and responds to rise in blood glucose by secreting insulin. Other stimuli like sight and taste of food, nerve stimulation and increased blood concentrations of other fuel molecules, including amino acids and fatty acids, also promote insulin secretion.

What happens when there is insufficient insulin?

Since insulin controls the central metabolic processes, failure of insulin production leads to a condition called diabetes mellitus. There are two major types of diabetes – type 1 and type 2.

Type 1 diabetes occurs when there is no or very low production of insulin from the pancreatic beta cells. Patients with Type 1 diabetes mellitus depend on external insulin (most commonly injected subcutaneously) for their survival.

In type 2 diabetes mellitus the demands of insulin are not met by the amount produced by the pancreatic beta cells. This is termed insulin resistance or "relative" insulin deficiency. These patients may be treated with drugs to reduce their blood sugar or may eventually require externally supplied insulin if other medications fail to control blood glucose levels adequately.

Diabetes, often referred to by doctors as **diabetes mellitus**, describes a group of metabolic diseases in which the person has high blood glucose (blood sugar), either because insulin production is inadequate, or because the body's cells do not respond properly to insulin, or both. Patients with high blood sugar will typically experience polyuria (frequent urination), they will become increasingly thirsty (polydipsia) and hungry (polyphagia). Diabetes is a long-term condition that causes high blood sugar levels.In 2013 it was estimated that over 382 million people throughout the world had diabetes. Type 1 Diabetes - the body does not produce insulin. Approximately 10% of all diabetes cases are type 1.Type 2 Diabetes - the body does not produce enough insulin for proper function. Approximately 90% of all cases of diabetes worldwide are of this type. Gestational Diabetes - this type affects females during pregnancy.

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The most common diabetes symptoms include **frequent urination**, intense thirst and hunger, weight gain, unusual weight loss, **fatigue**, cuts and bruises that do not heal, **male sexual dysfunction**, numbness and tingling in hands and feet.

If you have Type 1 and follow a healthy eating plan, do adequate exercise, and take insulin, you can lead a normal life. Type 2 patients need to eat healthily, be physically active, and test their blood glucose. They may also need to take oral medication, and/or insulin to control blood glucose levels. As the risk of cardiovascular disease is much higher for a diabetic, it is crucial that blood pressure and cholesterol levels are monitored regularly. As smoking might have a serious effect on cardiovascular health, diabetics should stop smoking. Hypoglycemia - low blood glucose - can have a bad effect on the patient. Hyperglycemia - when blood glucose is too high - can also have a bad effect on the patient. Some people may be able to control their type 2 diabetes symptoms by losing weight, following a healthy diet, doing plenty of exercise, and monitoring their blood glucose levels. However, type 2 diabetes is typically a progressive disease - it gradually gets worse - and the patient will probably end up have to take insulin, usually in tablet form.

If you have type 2 diabetes your body does not use insulin properly. This is called insulin resistance. At first, your pancreas makes extra insulin to make up for it. But, over time it isn't able to keep up and can't make enough insulin to keep your blood glucose at normal levels.

1. A) SYNTHESIS OF NATURAL INSULIN

Insulin is synthesized in significant quantities only in beta cells in the pancreas. Since it is a protein or a polypeptide structure it is synthesized like most other proteins via transcription and translation of DNA into mRNA and amino acid chains or polypeptide chains. Thereafter the protein undergoes structural changes to achieve its final form.

Steps in insulin synthesis

The insulin mRNA is translated as a single chain precursor called preproinsulin. Thereafter the 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
- a connecting peptide in the middle known as the C peptide

In the endoplasmic reticulum the proinsulin is exposed to several specific endopeptidases which excise the C peptide. This forms the mature form of insulin. Insulin and free C peptide are packed in the

Golgi bodies into secretory granules which accumulate in the cytoplasm

Insulin consists of two polypeptide chains, the A- and B- chains, linked together by disulfide bonds. It is however first synthesized as a single polypeptide called preproinsulin in pancreatic β-cells. Preproinsulin contains a 24-residue signal peptide which directs the nascent polypeptide chain to the rough endoplasmic reticulum (RER). The signal peptide is cleaved as the polypeptide is translocated into lumen of the RER, forming proinsulin. In the RER the proinsulin folds into the correct conformation and 3 disulfide bonds are formed. About 5–10 min after its assembly in the endoplasmic reticulum, proinsulin is transported to the trans-Golgi network (TGN) where immature granules are formed. Transport to the TGN may take about 30 min.

Proinsulin undergoes maturation into active insulin through the action of cellular endopeptidases known asprohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase E. The endopeptidases cleave at 2 positions, releasing a fragment called the C-peptide, and leaving 2 peptide chains, the B- and A-chains, linked by 2 disulfide bonds. The cleavage sites are each located after a pair of basic residues (lysine-64 and arginine-65, and arginine-31 and -32). After cleavage of the C-peptide, these 2 pairs of basic residues are removed by the carboxypeptidase.

The C-peptide is the central portion of proinsulin, and the primary sequence of proinsulin goes in the order "B-C-A" (the B and A chains were identified on the basis of mass and the C-peptide was discovered later).

The resulting mature insulin is packaged inside mature granules waiting for metabolic signals (such as leucine, arginine, glucose and mannose) and vagal nerve stimulation to be exocytosed from the cell into the circulation.

The endogenous production of insulin is regulated in several steps along the synthesis pathway:

- At transcription from the insulin gene
- In mRNA stability
- At the mRNA translation
- In the posttranslational modifications

1. B) SYNTHETIC HUMAN INSULIN

Synthetic human insulin was the first golden molecule of the biotech industry and the direct result of recombinant DNA technology. Currently, millions of diabetics worldwide use synthetic insulin to regulate their blood sugar levels. Synthetic insulin is made in both bacteria and yeast. Human insulin is the name which describes synthetic insulin which is laboratory grown to mimic the insulin in humans.

Human insulin was developed through the 1960s and 1970s and approved for

pharmaceutical use in 1982.Before human insulin was developed animal insulin, usually a purified form of porcine (pork) insulin, was used. Human insulin is laboratory created by growing insulin proteins within E-coli bacteria (Escherichia coli).

Human insulin is available in two forms, a short acting (regular) form and intermediate acting (NPH) form.NPH (Neutral Protamine Haledon) insulin, also known as isophane insulin, is a suspension meaning that the insulin vial should be rolled or repeatedly turned upside down to ensure the solution is uniformly cloudy.

Some examples of human insulin:

- **Regular (short acting):**Humulin S, Actrapid, Insuman Rapid
- **NPH (intermediate acting):** Humulin I, Insuman basal, Insulatard
- **Premixed human insulins:** Humulin M2, M3 and M5, Insuman Comb 15, 25 and 50

2)METHODOLOGY

The methods involved in writing this research paper is reading and researching about the topic using different sources like journals published, books and different websites providing information about the topic.

However, we have made sure to completely discuss the methods and the techniques used by the scientists to manufacture human insulin in vitro.

The main method used in the laboratory production of human insulin is GENETIC ENGINEERING and using the technique of RECOMBINANT DNA TECHNOLOGY.

In the 1980s, researchers used genetic engineering to manufacture human insulin. In 1982, the Eli Lilly Corporation produced human insulin that became the first approved genetically engineered pharmaceutical product.

Although bovine and porcine insulin are similar to human insulin, their composition is slightly different. Consequently, a number of patients' immune systems produce antibodies against it, neutralizing its actions and resulting in inflammatory responses at injection sites. Added to these adverse effects of bovine and porcine insulin, were fears of long term complications ensuing from the regular injection of a foreign substance, as well as a projected decline in the production of animal derived insulin. These factors led researchers to consider synthesizing *Humulin* by inserting the insulin gene into a suitable vector, the E. coli bacterial cell, to produce insulin that is chemically identical to its naturally

produced counterpart. This has been achieved using Recombinant DNA technology. This method is a more reliable and sustainable method than extracting and purifying the abattoir by-product.

Human insulin is grown in the lab inside common bacteria. *Escherichia coli* is by far the most widely used type of bacterium, but yeast is also used.

Researchers need the human protein that produces insulin. Manufacturers get this through an amino-acid sequencing machine that synthesizes the DNA. Manufacturers know the exact order of insulin's amino acids (the nitrogen-based molecules that line up to make up proteins). There are 20 common amino acids. Manufacturers input insulin's amino acids, and the sequencing machine connects the amino acids together. Also necessary to synthesize insulin are large tanks to grow the bacteria, and nutrients are needed for the bacteria to grow. Several instruments are necessary to separate and purify the DNA such as a centrifuge, along with various chromatography and x-ray crystallography instruments.

Synthesizing human insulin is a multi-step biochemical process that depends on basic recombinant DNA techniques and an understanding of the insulin gene. DNA carries the instructions for how the body works and one small segment of the DNA, the insulin gene, codes for the protein insulin. Manufacturers manipulate the biological precursor to insulin so that it grows inside simple bacteria.

1) The insulin gene is a protein consisting of two separate chains of amino acids, an A above a B chain that is held together with bonds. Amino acids are the basic units that build all proteins. The insulin A chain consists of 21 amino acids and the B chain has 30.

2) Before becoming an active insulin protein, insulin is first produced as preproinsulin. This is one single long protein chain with the A and B chains not yet separated, a section in the middle linking the chains together and a signal sequence at one end telling the protein when to start secreting outside the cell. After preproinsulin, the chain evolves into proinsulin, still a single chain but without the signaling sequence. Then comes the active protein insulin, the protein without the section linking the A and B chains. At each step, the protein needs specific enzymes (proteins that carry out chemical reactions) to produce the next form of insulin.

3) One method of manufacturing insulin is to grow the two insulin chains separately. This will avoid manufacturing each of the specific enzymes needed. Manufacturers need the two mini-genes: one that produces the A chain and one for the B chain. Since

the exact DNA sequence of each chain is known, they synthesize each mini-gene's DNA in an amino acid sequencing machine.

4) These two DNA molecules are then inserted into plasmids, small circular pieces of DNA that are more readily taken up by the host's DNA.

5) Manufacturers first insert the plasmids into a non-harmful type of the bacterium *E. coli*. They insert it next to the *lacZ* gene. LacZ encodes for 8-galactosidase, a gene widely used in recombinant DNA procedures because it is easy to find and cut, allowing the insulin to be readily removed so that it does not get lost in the bacterium's DNA. Next to this gene is the amino acid methionine, which starts the protein formation.

6) The recombinant, newly formed, plasmids are mixed up with the bacterial cells. Plasmids enter the bacteria in a process called transfection. Manufacturers can add to the cells DNA ligase, an enzyme that acts like glue to help the plasmid stick to the bacterium's DNA.

7) The bacteria synthesizing the insulin then undergo a fermentation process. They are grown at optimal temperatures in large tanks in manufacturing plants. The millions of bacteria replicate roughly every 20 minutes through cell mitosis, and each expresses the insulin gene.

8) After multiplying, the cells are taken out of the tanks and broken open to extract the DNA. One common way this is done is by first adding a mixture of lysozome that digest the outer layer of the cell wall, then adding a detergent mixture that separates the fatty cell wall membrane. The bacterium's DNA is then treated with cyanogen bromide, a reagent that splits protein chains at the methionine residues. This separates the insulin chains from the rest of the DNA.

9) The two chains are then mixed together and joined by disulfide bonds through the reduction-reoxidation reaction. An oxidizing agent (a material that causes oxidization or the transfer of an electron) is added. The batch is then placed in a centrifuge, a mechanical device that spins quickly to separate cell components by size and density.

10) The DNA mixture is then purified so that only the insulin chains remain. Manufacturers can purify the mixture through several chromatography, or separation, techniques that exploit differences in the molecule's charge, size, and affinity to water. Procedures used include an ion-exchange column, reverse-phase high performance liquid chromatography, and a gel filtration chromatography column. Manufacturers can test insulin batches to ensure none of the bacteria's *E. coli* proteins are mixed in with

the insulin. They use a marker protein that lets them detect *E. coli* DNA. They can then determine that the purification process removes the*E. coli* bacteria.

11) Starting in 1986, manufacturers began to use another method to synthesize human insulin. They started with the direct precursor to the insulin gene, proinsulin. Many of the steps are the same as when producing insulin with the A and B chains, except in this method the amino acid machine synthesizes the proinsulin gene.

12) The sequence that codes for proinsulin is inserted into the non-pathogenic *E. coli* bacteria. The bacteria go through the fermentation process where it reproduces and produces proinsulin. Then the connecting sequence between the A and B chains is spliced away with an enzyme and the resulting insulin is purified.

13) At the end of the manufacturing process ingredients are added to insulin to prevent bacteria and help maintain a neutral balance between acids and bases. Ingredients are also added to intermediate and long-acting insulin to produce the desired duration type of insulin. This is the traditional method of producing longer-acting insulin. Manufacturers add ingredients to the purified insulin that prolong their actions, such as zinc oxide. These additives delay absorption in the body. Additives vary among different brands of the same type of insulin

In the mid 1990s, researchers began to improve the way human insulin works in the body by changing its amino acid sequence and creating an analog, a chemical substance that mimics another substance well enough that it fools the cell. Analog insulin clumps less and disperses more readily into the blood, allowing the insulin to start working in the body minutes after an injection. There are several different analog insulin. Humulin insulin does not have strong bonds with other insulin and thus, is absorbed quickly. Another insulin analog, called Glargine, changes the chemical structure of the protein to make it have a relatively constant release over 24 hours with no pronounced peaks.

Instead of synthesizing the exact DNA sequence for insulin, manufacturers synthesize an insulin gene where the sequence is slightly altered. The change causes the resulting proteins to repel each other, which causes less clumping. Using this changed DNA sequence, the manufacturing process is similar to the recombinant DNA process described.

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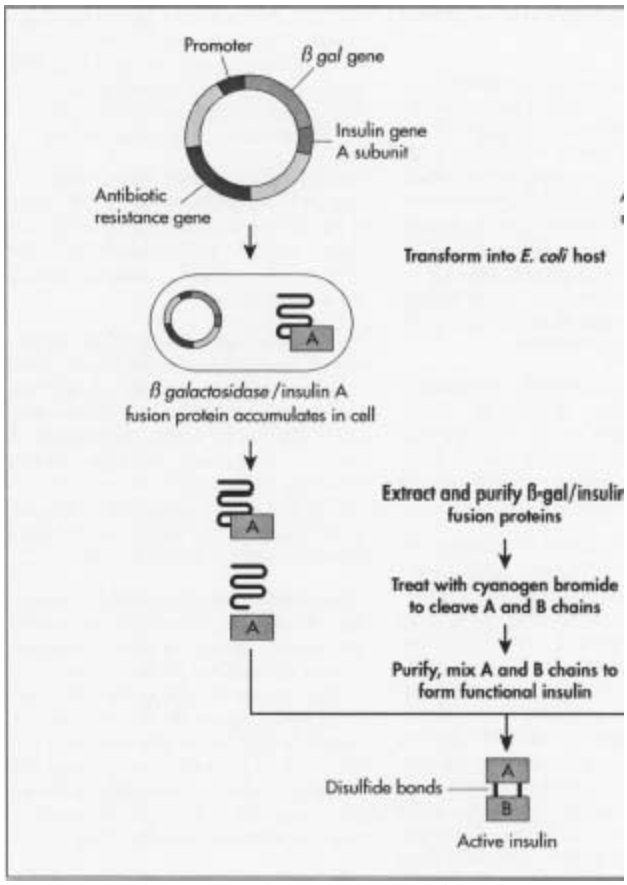

A diagram of the manufacturing steps for insulin.

3) RESULT:

Synthetic human insulin is largely regarded asa better substitute to animal insulin. It is less expensive and is absorbed more rapidly by the human body, it has shorter as well as moremanageable duration of effectiveness in the affected person and causes less allergic or autoimmune reactions than the animal insulin hormone.On other hand synthetic insulin has some of the side effects such as patient may experience extreme lethargy, mental confusion, memory loss, joint and muscle pains, depression, general feeling of beingunwell.

4) DISCUSSION:

Currently its not easy to say whether we should use synthetic insulin over animal insulin.While synthetic insulin provides more benefits thananimal insulin, people deem animal insulin a naturaltreatment, and therefore a better choice for their body inthe long run. Synthetic insulin is most widely prescribedand easier to come by, patients would like to be morereadily given the information and choice for animal insulin.

FUTUTRE SCOPE:

The future of insulin holds many possibilities. Since insulin was first synthesized, diabetics needed to regularly inject the liquid insulin with a syringe directly into their bloodstream. This allows the insulin to enter the blood immediately.

Insulin in future can be very helpful as now the technology has became so advanced so we can produce several ways of injecting insulin in human body.Use of synthetic human insulin has also reduced the reliability on animal insulin as synthetic human insulin is much more beneficial than the animal insulin.

The future of insulin holds many possibilities. Since insulin was first synthesized, diabetics needed to regularly inject the liquid insulin with a syringe directly into their bloodstream. This allows the insulin to enter the blood immediately. For many years it was the only way known to move the intact insulin protein into the body. In the 1990s, researchers began to make inroads in synthesizing various devices and forms of insulin that diabetics can use in an alternate drug delivery system.

Manufacturers are currently producing several relatively new drug delivery devices. Insulin pens look like a writing pen. A cartridge holds the insulin and the tip is the needle. The user set a dose, inserts the needle into the skin, and presses a button to inject the insulin. With pens there is no need to use a vial of insulin. However, pens require inserting separate tips before each injection. Another downside is that the pen does not allow users to mix insulin types, and not all insulin is available.

For people who hate needles an alternate to the pen is the jet-injector. Looking similar to

the pens, jet injectors use pressure to propel a tiny stream of insulin through the skin. These devices are not as widely used as the pen, and they can cause bruising at the input point.

The insulin pump allows a controlled release in the body. This is a computerized pump, about the size of a beeper, that diabetics can wear on their belt or in their pocket. The pump has a small flexible tube that is inserted just under the surface of the diabetic's skin. The diabetic sets the pump to deliver a steady, measured dose of insulin throughout the day, increasing the amount right before eating. This mimics the body's normal release of insulin. Manufacturers have produced insulin pumps since the 1980s but advances in the late 1990s and early twenty-first century have made them increasingly easier to use and more popular. Researchers are exploring the possibility of implantable insulin pumps. Diabetics would control these devices through an external remote control.

Researchers are exploring other drug-delivery options. Ingesting insulin through pills is one possibility. The challenge with edible insulin is that the stomach's high acidic environment destroys the protein before it can move into the blood. Researchers are working on coating insulin with plastic the width of a few human hairs. The coverings would protect the drugs from the stomach's acid.

In 2001 promising tests are occurring on inhaled insulin devices and manufacturers could begin producing the products within the next few years. Since insulin is a relatively large protein, it does not permeate into the lungs. Researchers of inhaled insulin are working to create insulin particles that are small enough to reach the deep lung. The particles can then pass into the bloodstream. Researchers are testing several inhalation devices much like that of an asthma inhaler.

Another form of aerosol device undergoing tests will administer insulin to the inner cheek. Known as buccal (cheek) insulin, diabetics will spray the insulin onto the inside of their cheek. It is then absorbed through the inner cheek wall.

Insulin patches are another drug delivery system in development. Patches would release insulin continuously into the bloodstream. Users would pull a tab on the patch to release more insulin before meals. The challenge is finding a way to have insulin pass through the skin. Ultrasound is one method researchers are investigating. These low frequency sound waves could change the skin's permeability and allow insulin to pass.

Other research has the potential to discontinue the need for manufacturers to synthesize insulin. Researchers are working on creating the cells that produce insulin in the laboratory. The thought is that physicians can someday replace the non-working pancreas cells with insulin-producing cells. Another hope for diabetics is gene therapy. Scientists are working on correcting the insulin gene's mutation so that

diabetics would be able to produce insulin on their own.

APPLICATIONS AND USE:

One of the advantages of producing insulin using the recombinant DNA method is to reduce the dependency on animal glands. Also, by using insulin that chemically is identical to human insulin, scientists hope that certain allergic reactions by some diabetes to insulin derived from animals can be eliminated.

The development of human insulin demonstrates the viability of using recombinant DNA technology to produce products with practical application.

Human insulin has increased purity as compared with extractive animal insulin, it has enhanced purity which reduces antibody formation.

Human insulin is the only animal protein to have been made in bacteria in such a way that the structure is absolutely identical to that of the natural molecule.

This reduces the possibility of complications resulting from antibody production. In chemical and pharmacological studies, commercially available recombinant DNA human insulin has proven undistinguishable from pancreatic human insulin. Initially the major difficulty which was seen was the contamination of the final product by the host cells , increasing the risk of contamination in the fermentation broth. The danger was eradicated by the introduction of purification processes. When the final insulin product is subjected to a battery of tests, including the finest radio-immuno assay techniques, no impurities can be detected. The entire procedure is performed using yeast cells as a growth medium, as they secrete an almost complete human insulin molecule with perfect three dimensional structure .This minimizes the need for complex and costly purification procedures.

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