Insulin Production

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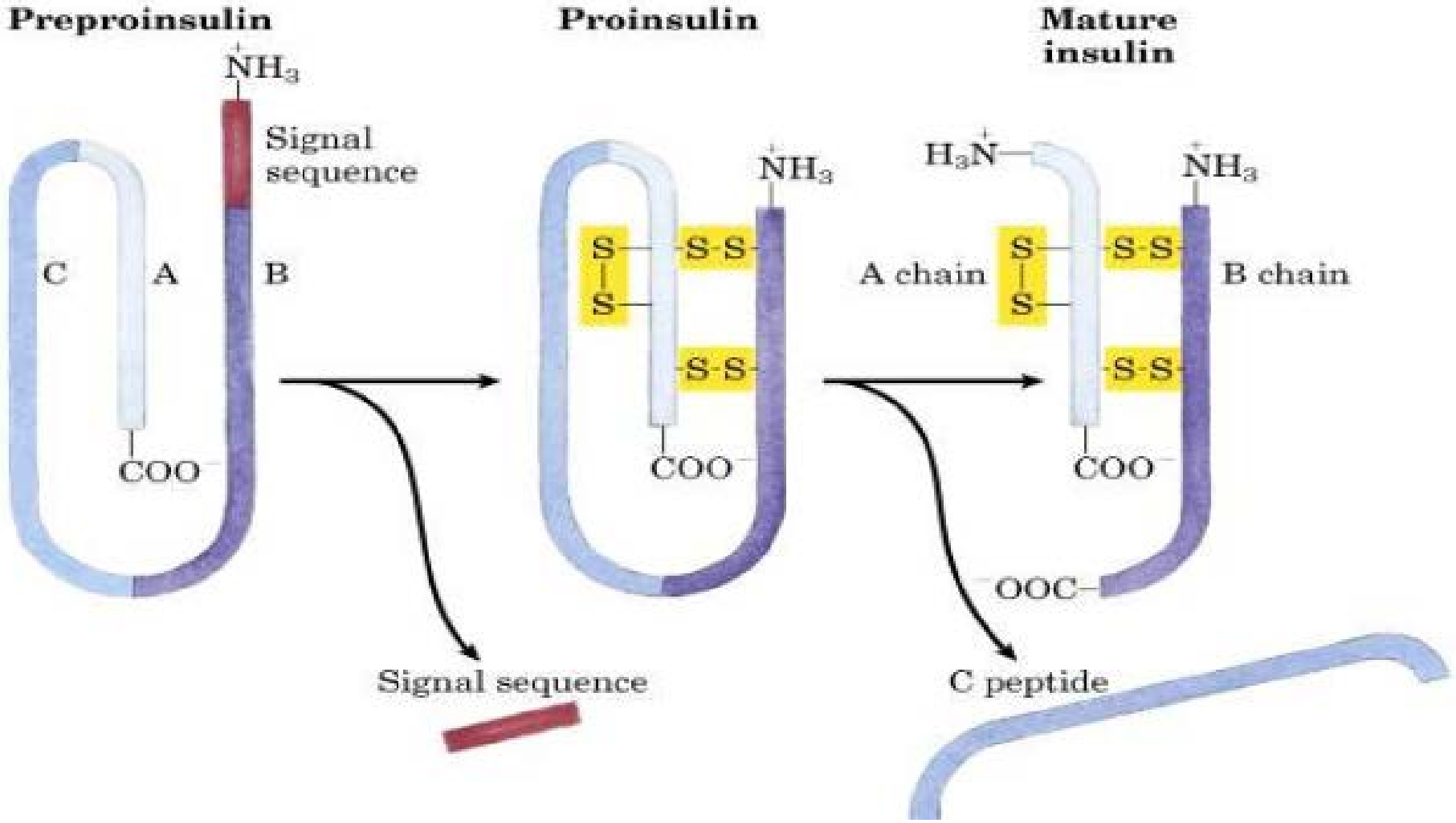
Structure and function of insulin

- The human insulin is comprised of 51 amino acids and has a mol. weight of 5808 Da.
- It is produced by beta cells of the pancreas and plays a key role in regulating carbohydrate and fat metabolism in the body.
- Insulin is synthesized as a single polypeptide (preproinsulin) in pancreatic beta cells.
- Preproinsulin harbours a 24-residue signal peptide, which directs the nascent polypeptide to the endoplasmic reticulum.
- The signal peptide is cleaved as the polypeptide is translocated into the endoplasmic reticulum resulting in the formation of proinsulin.
- In the Endoplasmic reticulum, the proinsulin is folded in proper confirmation with the formation of 3 disulphide bonds.
- Folded proinsulin is then transported to the trans-Golgi network, where it is converted into active insulin by cellular endopeptidases and exoprotease carboxypeptidase E.
- The endopeptidases cleaves at two positions, resulting in the release of a fragment termed as C-peptide.
- The mature insulin, thus formed consists of an A-chain with 21 aminoacids and a B-chain containing 30 aminoacids and both polypeptides linked together by two disulphide bonds.
- Besides, the A-chain has an intrachain disulphide bond.

Structure of insulin.
Preproinsulin – Proinsulin – Insulin



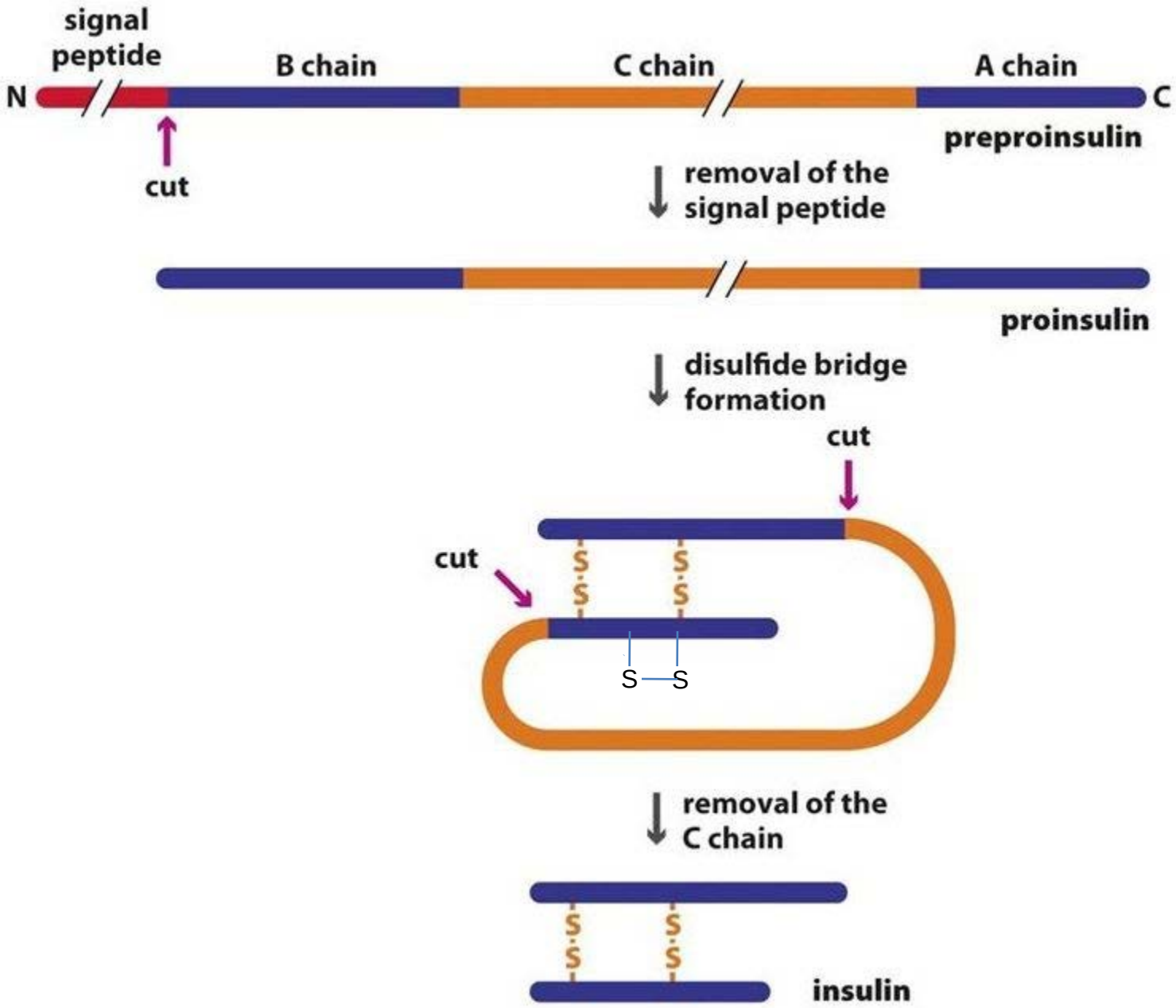


Figure 8.24 Introduction to Genetics (© Garland Science 2012)

rDNA technology in insulin production

- Gene encoding human insulin was cloned and expressed in *E. coli* in 1978.
- The first licensed drug produced using recombinant DNA technology was human insulin (Humulin), which was developed by Genentech and licensed as well as marketed by Eli Lilly in 1982.
- Since the early 1920s, diabetic patients were treated with insulin, which was purified from bovine or porcine pancreas.
- Nowadays, recombinant human insulin is mainly produced either in *E. coli* or *Saccharomyces cerevisiae*.
- Using *E. coli* expression system, the insulin precursors (IP) are produced as inclusion bodies and fully functional polypeptides are obtained finally by solubilization and refolding procedures.
- Yeast based expression system yield soluble IP which is secreted into the culture supernatant.

Analogue Insulin

- The first generation recombinant insulins have an amino acid sequence identical to native human insulin and are preferred over animal derived insulin products.
- However, advancement in the field of genetic engineering and development of technology to chemically synthesize genes with altered nucleotide sequence, facilitated the development of insulin analogues with altered amino acid sequence.
- It had been observed that native insulin in commercial preparations usually exist in oligomeric form, as zinc-containing hexamer due to very high concentration, but in blood, biologically active insulin is in monomeric form.
- Hence, in order to develop a fast- acting insulin analogue, it was required to modify the amino acids residues whose side chains are involved in dimer or oligomer formation.
- To avoid multiple injection, long-acting insulin analogues with prolonged duration of actions have also generated. Eg. Glargine insulin
- Glargine was generated by replacing the C-terminal asparagine of the A-chain with a glycine residue and the C-terminal of the B- chain was modified by adding two arginine residues.

Expression of Insulin gene & production in *E. coli*

Approach I (Two chain method)

- 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*.
- 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.

Approach II (Proinsulin method)

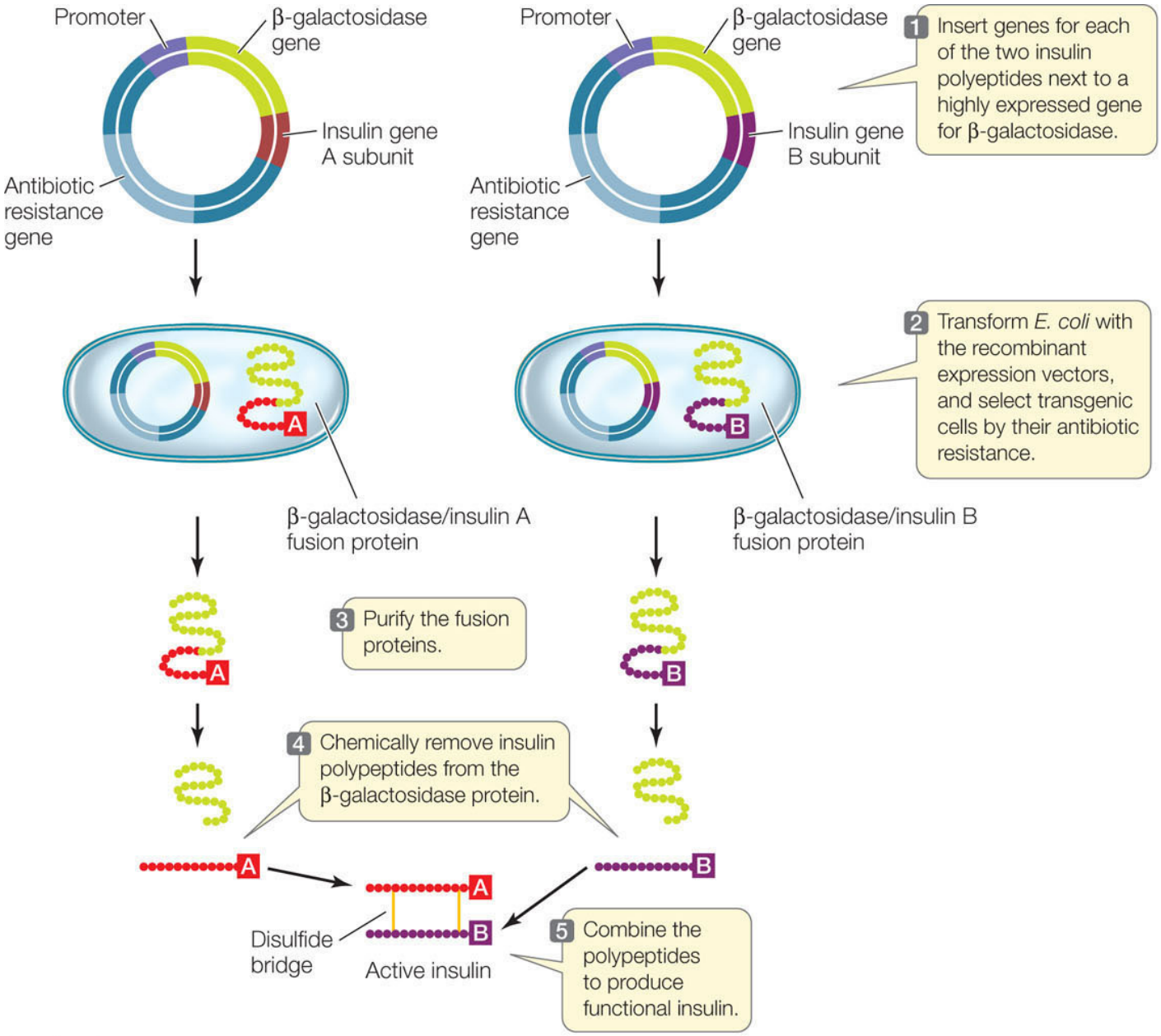
- Another approach involves the expression of a single chemically synthesized cDNA encoding for human proinsulin in *E. coli* followed by purification and subsequent excision of C-peptide by proteolytic digestion.
- This approach was more efficient and convenient for large scale production of therapeutic insulin as compared to the two chain combination approach and has been used commercially since 1986.

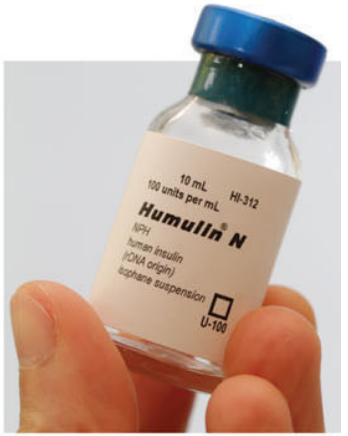
The Manufacturing Process through Approach I

- 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 a DNA synthesizer machine.
- These two DNA molecules are then inserted into plasmids, next to the *lacZ* gene.
- LacZ encodes for β-galactosidase, a gene widely used in recombinant DNA procedures because it is easy to find and cut, allowing the insulin to be readily removed.
- The resulting combination protein products are called fusion proteins.
- Next to this gene is the amino acid methionine, which starts the protein formation.
- The recombinant, newly formed, plasmids are mixed up with the bacterial cells.
- Plasmids enter the bacteria in a process called transfection.
- The bacteria synthesizing the insulin (fusion protein) 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.

...The Manufacturing Process through Approach I

- After multiplying, the cells are taken out of the tanks and broken open to extract the fusion protein.
- 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 fusion protein 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 peptide chain.
- 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, to separate cell components by size and density.
- The protein 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* proteins.
- They can then determine that the purification process removes the *E. coli* bacteria.





13.12: David McIntyre.

The Manufacturing Process through Approach II

- Many of the steps are the same as when producing insulin with the A and B chains, except in this method the DNA synthesizer machine synthesizes the proinsulin gene.
- The sequence that codes for proinsulin is inserted into the *E. coli* bacteria.
- The bacteria go through the fermentation process where it reproduces and produces proinsulin as a recombinant fusion protein within microbial cells.
- Proinsulin cleaved out by treatment with cyanogen bromide.
- The formation of native insulin from proinsulin follows two main steps:
  - folding & formation of disulfide bridges within the proinsulin molecule, and
  - proteolytic cleavage (trypsin & carboxypeptidase B) with subsequent release of the connecting C-peptide.
- 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.

… Expression of Insulin gene & production in *E. coli*

- Intracellular overexpression of insulin chain A, B or proinsulin as a fusion protein product (up to 25-30% of total cell protein in *E. coli* cells) induces the formation of inclusion bodies, insoluble aggregates of the recombinant protein product.
- The insoluble form of the protein product protects proinsulin from degradation by proteolysis within the microbial cells.
- The aggregated form of the proteins of interest also facilitates separation of these aggregates from other cell debris via centrifugation after cell disruption, due to the dense nature of the inclusion bodies.
- However, other process design complexities accompany this type of intracellular expression.
- For example, solubilization of the inclusion bodies and renaturation processes are required during downstream processing in order to isolate the discrete insulin chain A, B or proinsulin fusion proteins from the inclusion bodies for processing and purification.

Fermentation for insulin production using *E. coli* cells

- The first step of the process is to grow enough of the insulin chain A, B or proinsulin producing *E. coli* bacteria so as to acquire a sufficient amount of insulin per process.
- In order to do this an original amount of *E. coli* cells containing the plasmid for insulin chains or proinsulin production will be grown in test tubes containing tryptic soy broth and kanamycin monosulfate.
- Within this mixture the tryptic soy broth provides nutrients for the *E. coli* while the kanamycin monosulfate acts to kill any bacteria within the mixture which was not given kanamycin resistance; the plasmid containing the insulin coding gene also contains gene coding for kanamycin resistance.
- Once the growth mixture contains only the growth media and *E. coli* carrying the plasmid for insulin production, it is desired that the *E. coli* cell count be increased and to initiate production of the insulin inclusion bodies.
- All of this is accomplished by placing the original growth mixture into a bioreactor in which the parameters can be controlled for maximum cell growth and insulin production.
- Within a bioreactor the temperature, pH, foam, and feed can be controlled automatically to yield maximum results.
- For E. coli the best growth conditions are that of a pH of 7 and a temperature of 37°C.

Reagents Involved:

1L inoculation solution
25mL NH_3
30mL H_3PO_4
100mL 87% glycerol feed
25g $(NH_4)_2SO_4$
30G $KH_2PO_4*H_2O$
20G $KHPO_4*2H_2O$
5g Na3-citrate
10g yeast extract
0.7g thiamine
10mL trace element solution
6.5mL vitamin solution
10mL adecanol LG-109 (antifoam)
10mL B-indole acrylic acid
Water up to 10L total

Parameters:

10L total working volume
31 hour growth phase
pH 7
37 C

Yeast expression system for the production of insulin

- The therapeutic proteins produced in yeast are specifically from Saccharomyces cerevisiae and include hormones (insulin, insulin analogues, non-glycosylated human growth hormone somatotropin, glucagon), vaccines (hepatitis B virus surface antigen) etc.
- Very high level of expression of heterologous proteins can be attained in *Pichia pastoris*, that might constitute about 30% of total cellular protein which is very high as compared to *S. cerevisiae*.
- Therefore, *Pichia 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, the overall production space-time yield remains similar.

... Yeast expression system for the production of insulin

- *Saccharomyces cerevisiae* has been extensively used to produce recombinant human insulin since early 1980s and a large proportion of recombinant commercial insulins are produced by this yeast expression system.
- For efficient expression and secretion of recombinant proinsulin in yeast, insulin construct was engineered to contain the native A-chain and a B-chain lacking the C-terminal B30 threonine, either directly fused or linked via a short synthetic C peptide.
- The cDNA sequence encoding for this construct was fused with α-factor signal sequence of *Saccharomyces cerevisiae* for secreted expression of proinsulin which gave yield upto 80 mg/ml of insulin.
- The single chain proinsulin was purified and converted to active insulin by a trypsin-mediated transpeptidation reaction in presence of threonine ester.
- Besides native recombinant insulin, various insulin analogues are also being produced in *S. cerevisiae*.

Questions

- What are the approaches used for insulin production using *E. coli*?
- Discuss fermentation systems used for human insulin production.
- Diagrammatically represent the industrial production of human insulin.