# PRODUCTION OF BIOPHARMACEUTICALS

# WHAT ARE BIOPHARMACEUTICALS

- Biologically significant compounds like hormones and proteins useful for treatment of variety of human health disorders, usually called as Biopharmaceuticals or Biotherapeutics or Biologicals
- They are usually obtained from biological source and produced through industrial biotechnology
- commercial production of recombinant Biologicals and therapeutics now became an important area in global industralization
- The process developed in production of therapeutics has to be taken to industrial scale which is mainly done through fermentation technology

# FROM GENES TO PROCESSES

Gene discovery

↓

Cloning/expression

↓

Production and scale-up

↓

Engineering the protein

↓

Application

Pilot scale Fermenter

# Which Microbes are useful

Several species belonging to the following categories of microorganisms are useful

PROKARYOTIC Unicellular: Bacteria, Cyanobacteria
Multicellular: Cyanobacter

EUKARYOTIC Unicellular: Yeasts, Algae
Multicellular: Fungi, Algae

# SOME APPROVED THERAPEUTICS

The products of rDNA technology

- Hormones
  - Growth hormone, Insulin, Calcitonin, FSH
- Proteins:
  - EPO, CSF, Neurotrophic factors
- Cytokines
  - Interferons (Interleukins)
- Clotting factors
  - Factor VIII, Factor IX
- Vaccines
  - Hepatitis B, acellular pertussis vaccine (*Bordatella pertussis*, whooping cough)
- Monoclonal antibodies

# Commercial Production of Some important Biotherapeutics

# Insulin - first recombinant protein to be produced

- Insulin is an important hormone which regulates sugar metabolism
- An inability to produce insulin results in a form of diabetes, this disease can be treated by daily injections of insulin
- Historically, insulin from pigs or cows is used, but known to produce immune reactions in some patients
- Challenge: how to make human insulin to be used as a drug in cell systems or microbes?

# HISTORY OF INSULIN PRODUCTION

- Insulin biotechnology started by Genentech company in 1978 and it was marketed by Eli Lilly in 1982

- The human gene responsible for insulin production is cut from human DNA and inserted into a *plasmid* to make *recombinant DNA*.

- The recombinant DNA is inserted into bacteria cells which are *transformed* and begin producing insulin

# MANUFACTURING OF INSULIN

- The insulin gene is translated to a protein consisting of two separate chains of amino acids, an A above a B chain, that are held together with disulfide bonds
- The insulin A chain consists of 21 amino acids and the B chain has 30 with a molecular weight of 5808 Da
- 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 protease enzymes to produce the next form of insulin

# MANUFACTURING OF INSULIN

•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.

•These two DNA molecules are then inserted into plasmids, that are more readily taken up by the host's DNA.

•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 β-galactosidase 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

# MANUFACTURING OF INSULIN

- 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.

- 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.

- 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.

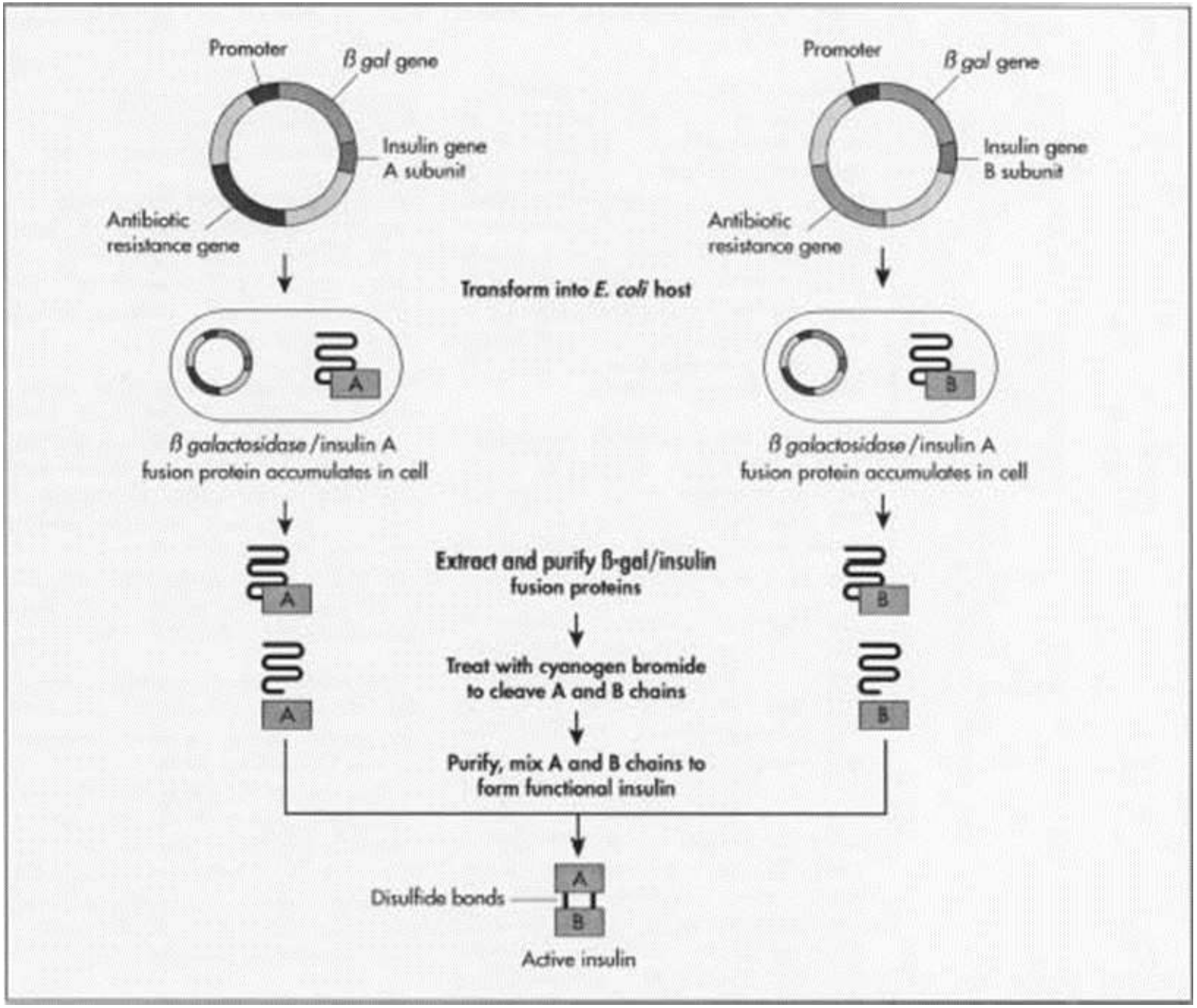
- The bacterium's DNA is then treated with cyanogen bromide, a reagent that splits protein chains at the methionine residues. This separates the insulin chains from the rest of the DNA.

# MANUFACTURING OF INSULIN

- The two chains are then mixed together and joined by disulfide bonds through the reduction-reoxidation reaction. An oxidizing agent is added. The batch is then placed in a centrifuge to separate cell components by size and density.

- The DNA mixture is 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.


Promoter
ß gal gene
Insulin gene
A subunit
Antibiotic
resistance gene
Promoter
ß gal gene
Insulin gene
B subunit
Antibiotic
resistance gene
Transform into E. coli host
A
B
ß galactosidase/insulin A
fusion protein accumulates in cell
ß galactosidase/insulin A
fusion protein accumulates in cell
A
A
B
B
Extract and purify ß-gal/insulin
fusion proteins
Treat with cyanogen bromide
to cleave A and B chains
Purify, mix A and B chains to
form functional insulin
Disulfide bonds
A
B
Active insulin

# MANUFACTURING OF PROINSULIN

•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 A diagram of the manufacturing steps for insulin.

- 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. Saccharomyces cerevisiae is the most preferred and predominant yeast for large scale commercial production of insulin
- Besides, E.coli and yeast, mammalian cells, transgenic animals and plant expression systems are also employed as a host for large-scale production of recombinant insulin

# PRODUCTION OF IP

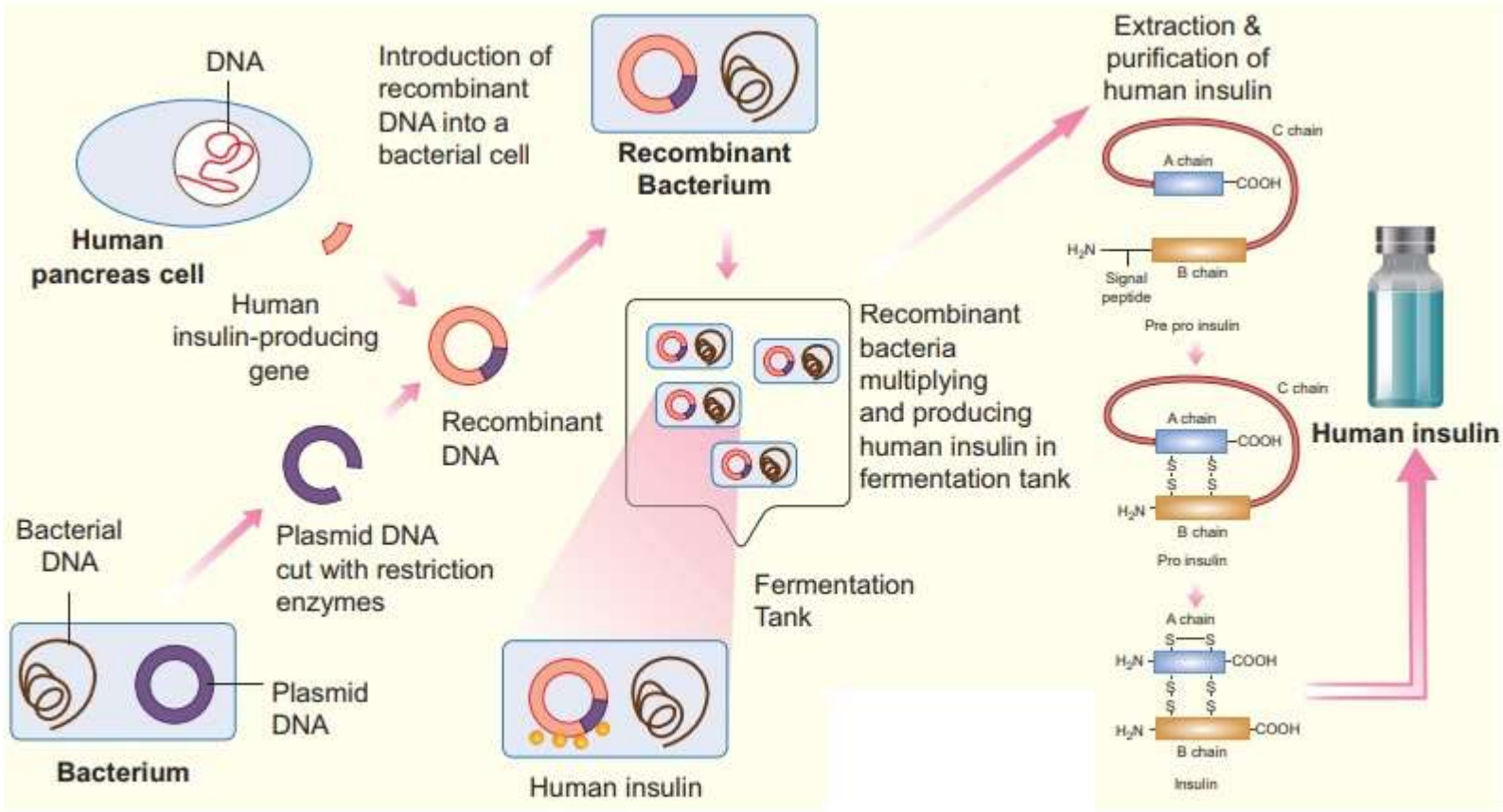



**Fig. 10.1 Human Insulin Production**

# E. COLI AS EXPRESSION ORGANISM

- Escherichia coli has always been preferred for production of recombinant proteins as it offered several advantages including high growth rate, simple media requirement, easy to handle, high yield and very cost effective.
- Disadvantages using E. coli expression system includes loss of plasmid and antibiotic property, unsolicited inducers for gene expression, intracellular accumulation of heterologous proteins as inclusion bodies, improper protein refolding, lack of post- translational modifications, protein-mediated metabolic burden and stress, endotoxin contamination, poor secretion, proteolytic digestion and complexity in downstream process

# MODIFICATION TO E. COLI TO IMPROVE EXPRESSION

- 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

- Engineering of Campylobacter jejuni N-linked glycosylation pathway into E. coli, provides an opportunity to express heterologous proteins in glycosylated form in E. coli.

# MODIFICATION TO E. COLI TO IMPROVE EXPRESSION

- the frequency of the codon usage reflects the abundance of their corresponding tRNA. Therefore, significant differences in codon usage could result in premature termination of translation, misincorporation of amino acids and inhibition of protein synthesis.
- Expression of heterologous proteins in E. coli can be improved by replacing codons that are rarely found in highly expressed E. coli genes with more favorable major codons.
- Co-expression of the genes encoding for a number of the tRNA for rare codon, may enhance the expression of heterologous proteins in E. coli. There are some commercial E. coli strains available that encodes for tRNA for rare codons such as BL21 (DE3) CodonPlus-RIL, BL21 (DE3) CodonPlus- RP and Rosetta (DE3). BL21 (DE3) CodonPlus-RIL harbors tRNA genes for rare codons like AGG, AGA (arginine), AUA (isoleucine) and CUA (leucine). Similarly, Rosetta (DE3) strain harbors tRNA genes for rare codons like AGG, AGA (arginine), CGG (arginine), AUA (isoleucine), CUA (leucine), CCC (proline) and GGA (glycine).

# MODIFICATION TO E. COLI TO IMPROVE EXPRESSION

- The use of protease-deficient E. coli strains, which carry mutations that eliminate the production of proteases may improve the yield of the therapeutic protein by reducing proteolytic degradation.
- *E. coli* strain BL-21, is deficient in two proteases which makes it suitable for expression of many proteins

# YEAST AND MAMMALIAN CELLS AS EXPRESSION SYSTEM

- Among yeast strains, Saccharomyces cerevisiae, Hansenulla polymorpha and Pichia pastoris are very commonly used for production of recombinant proteins
- Like E. coli, they grow rapidly and are very easy to handle and amenable to various genetic manipulations.
- Recombinant proteins produced in yeast are properly folded and glycosylated to a certain extent similar to the one expressed in mammalian cells.
- Various human therapeutic proteins, including monoclonal antibodies are being produced in mammalian cell lines such as Chinese hamster ovary (CHO) and Baby hamster kidney (BHK) cells.
- Recombinant proteins expressed in mammalian cells are properly folded, glycosylated and generally yield a functionally active protein but the production cost is considered very high due to expensive culture media.

# QUALITY CONTROL

- After synthesizing the human insulin, the structure and purity of the insulin batches are tested through several different methods.

- High performance liquid chromatography is used to determine if there are any impurities in the insulin. Other separation techniques, such as X-ray crystallography, gel filtration, and amino acid sequencing, are also performed. Manufacturers also test the vial's packaging to ensure it is sealed properly.

- Manufacturing for human insulin must comply with National Institutes of Health procedures for large-scale operations. The United States Food and Drug Administration must approve all manufactured insulin

# MODIFICATION OF INSULIN BY BIOTECHNOLOGY

- 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.

- Instead of synthesizing the exact DNA sequence for insulin, manufacturers synthesize an insulin gene where the sequence is slightly altered leading to change in the physicochemical properties of the resulting protein

# MODIFICATION OF INSULIN BY BIOTECHNOLOGY IN E.COLI

- **Insulin Lispro:** the first fast acting insulin analogue to obtain regulatory approval in 1996, for therapeutic use
- Insulin Lispro is engineered to have similar amino acid sequence as the native insulin but has an inversion of proline-lysine sequence at position 28 and 29 of the B-chain, which resulted in reduced hydrophobic interactions and thus prevented dimer formations.
- **Glulisine (Apidra):** rapid acting insulin approved by US regulatory authorities in 2004.
- Insulin Glulisine have been generated by replacing B3 asparagine by a lysine and B29 lysine replaced by glutamic acid

# MODIFICATION OF INSULIN BY BIOTECHNOLOGY IN E.COLI

- **Insulin Glargine** is one of long-acting insulin analogues, which was approved by regulatory authorities of USA and EU in 2000.
- 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.
- These modifications resulted in increase of the isoelectric point (pI) from 5.4 to neutral values.
- Glargine was finally formulated at pH 4 in soluble form.
- After SC administration, it precipitated due to neutral pH in the SC tissue. Resolubilization of insulin occur slowly, resulting in longer duration for its release in the blood

# MODIFICATION OF INSULIN BY BIOTECHNOLOGY IN S. CEREVISIAE

- **Insulin Aspart** is another fast-acting insulin analogue, which was produced in S. cerevisiae, approved by US FDA in 2001 for therapeutic use in human.
- Insulin Aspart was generated by replacing proline residue at position 28 with aspartic acid in the B-chain.
- This genetic modification resulted in an increase in inter-chain charge repulsion, decrease in self-association and thus causing rapid entry into the blood from the site of subcutaneous injection

# MODIFICATION OF INSULIN BY BIOTECHNOLOGY IN S. CEREVISIAE

- **Insulin Detemir** is another recombinant long-acting insulin analogue that was commercially produced in S. cerevisiae, approved for therapeutic use in human in 2004 by European regulatory authorities.
- Recombinant Detemir have been generated by removing the threonine residue at the 30 position of the B-chain, and a C14 fatty acid chain covalently attached to the lysine residue at the 29 position of the B-chain.
- These genetic alterations resulted in the binding of insulin to albumin in plasma, which ensured the slow and constant release of insulin and thus prolonging its duration of action up to 24 hours

# ADDITIVES TO PROLONG THE HALF LIFE

- 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.

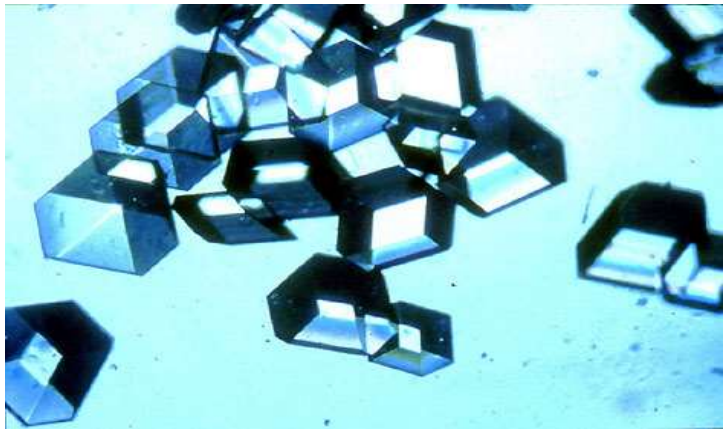
# Reverse transcription: cDNA Cloning

Suppose we need to clone a gene containing lots of introns. What will happen when the bacterium tries to express it?

To overcome this problem, we can start with mRNA isolated from tissues that produce the desired protein. We then use reverse transcriptase enzyme (produced by a retrovirus related to HIV) to reverse transcribe the mRNA into a DNA molecule that now is free of introns. Now we can ligate "sticky ends" onto the cDNA and recombine it into a phage or plasmid vector.

Or: Use Proteins sequence - BLAST website to get DNA sequence and PCR to make multiple copies of cDNA that can then be cloned.

# Production of Human Insulin by Bacteria

# HUMAN GROWTH HORMONE (HGH, OR SOMATOTROPIN)

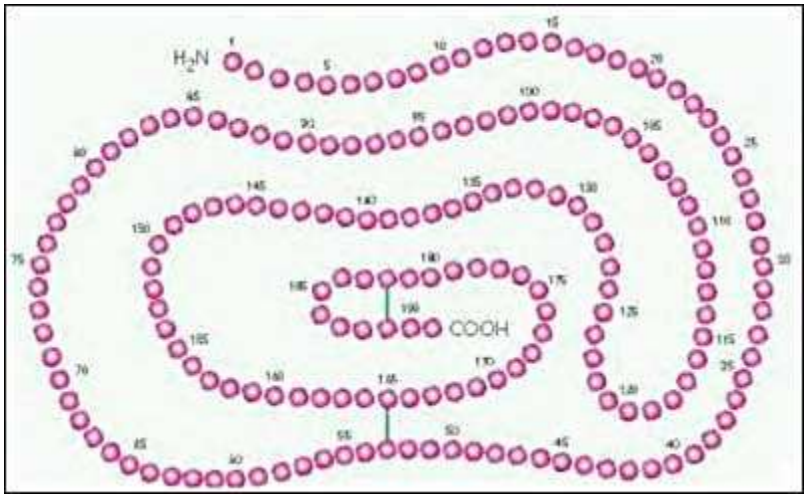
- Secreted by the pituitary gland, and is responsible for normal body growth and development, by stimulating protein production in muscle cells, energy release from the breakdown of fats and stimulates the development of bones

- These processes together are responsible for longitudinal growth. Inadequate production of GH results in short stature, defined as a below normal height for a given age

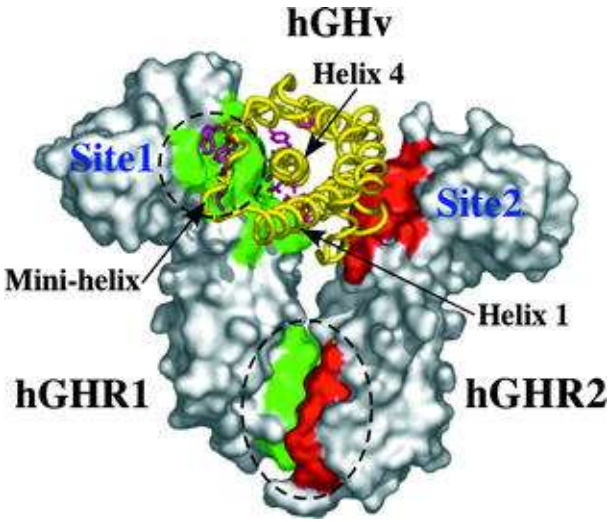
# HUMAN GROWTH HORMONE (HGH)

- In children and adolescents, the rate of growth in height is primarily determined by the rate at which endogenous GH is secreted
- The growth spurt during puberty is caused by increased secretion of GH
- Under normal conditions, GH secretion and growth rate remain increased until final height is reached, after which GH secretion is reduced to a steady state

# Structure of human Growth hormone

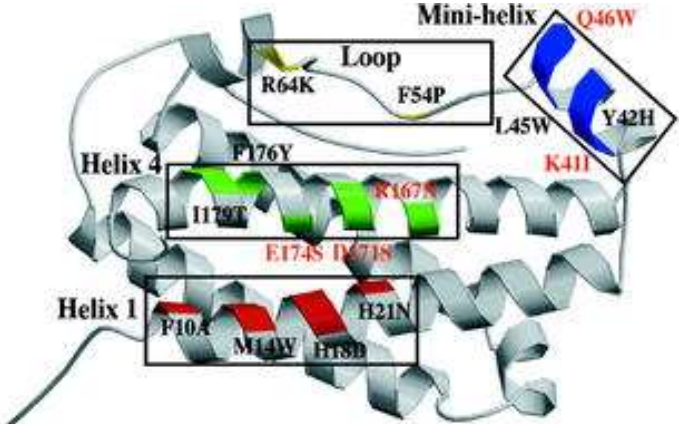



Primary structure




Hormone binding to receptors

**GROWTH HORMONE: 191 AMINO ACIDS, SINGLE CHAIN**




Teritiary structure

# PRODUCTION OF RECOMBINANT HGH

Isolating and constructing hGH cDNAs

Constructing expression cassette with hGH cDNAs inserts

Cultivating the recombinant clones in small scale flask/bioreactor

Producing the hGH in pilot scale bioreactors

Developing large scale purification procedure and process chromatography optimization (Affinity chromatography)

# PRODUCTION OF HGH

- Purification of recombinant human growth hormone (rhGH) from Chinese hamster ovary (CHO) cell culture supernatant by Gradiflow large-scale electrophoresis is described.
- Production of rhGH using *E. coli* as an alternative for using CHO cells, with the advantage of CHO cells that rhGH is secreted into protein-free production media, facilitating a more simple purification and avoiding resolubilization of inclusion bodies and protein refolding proteins when expressed in E. coli cells

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PLOS ONE

# Complete Solubilization and Purification of Recombinant Human Growth Hormone Produced in *Escherichia coli*

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## Abstract

High-level expression of recombinant human growth hormone (hGH) in *Escherichia coli (E. coli)* leads to the formation of insoluble aggregates as inclusion bodies devoid of biological activity. Until recently, significant efforts have been made to improve the recovery of active hGH from inclusion bodies. Here, we developed an efficient procedure for the production of completely soluble hGH by minimizing the formation of inclusion bodies and optimizing protein purification conditions. Under the newly established conditions we were able to obtain most of the total hGH in the soluble fraction. We show that the soluble protein can be efficiently purified in high yield by a series of chromatographic procedures. We analyzed the resulting hGH using various analytical techniques such as reversed-phase high-performance liquid chromatography (RP-HPLC), size-exclusion chromatography (SEC), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and circular dichroism (CD). These multiple analyses support the conclusion that we obtained highly pure hGH with the expected molecular mass and intact secondary structure. The biological activity of purified hGH was also confirmed by evaluating its growth-promoting effect using a cell proliferation assay. Taken together, we describe a straightforward strategy for the production of completely soluble and biologically active hGH in *E. coli*.

**Citation:** Kim M-J, Park HS, Seo KH, Yang H-J, Kim S-K, et al. (2013) Complete Solubilization and Purification of Recombinant Human Growth Hormone Produced in *Escherichia coli*. PLoS ONE 8(2): e56168. doi:10.1371/journal.pone.0056168

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**Competing Interests:** The authors have declared that no competing interests exist.

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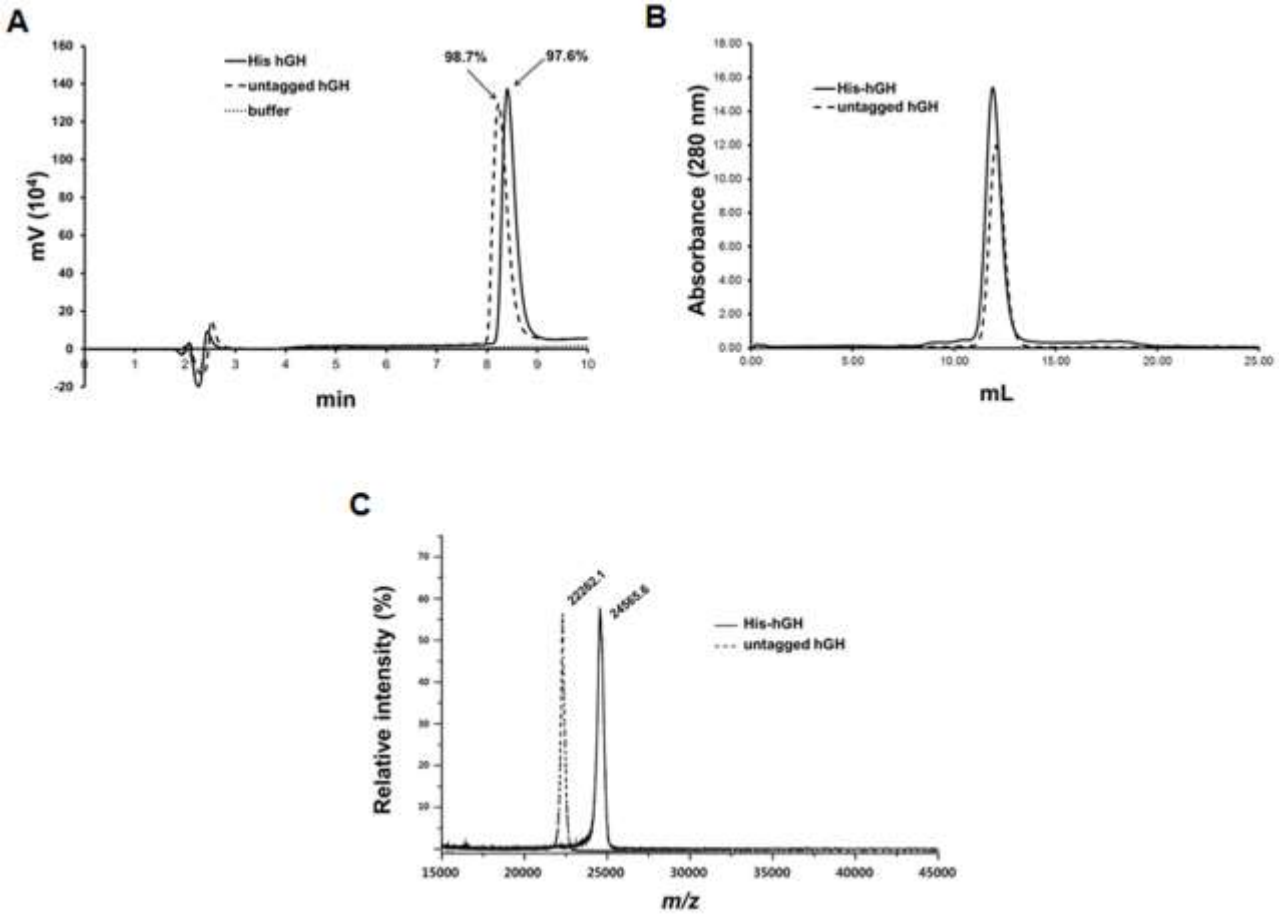
☯ These authors contributed equally to this work.

# SEPARATION OF HIS-TAGGED VS UNTAGGED HGH



# Strategy for production of growth hormone

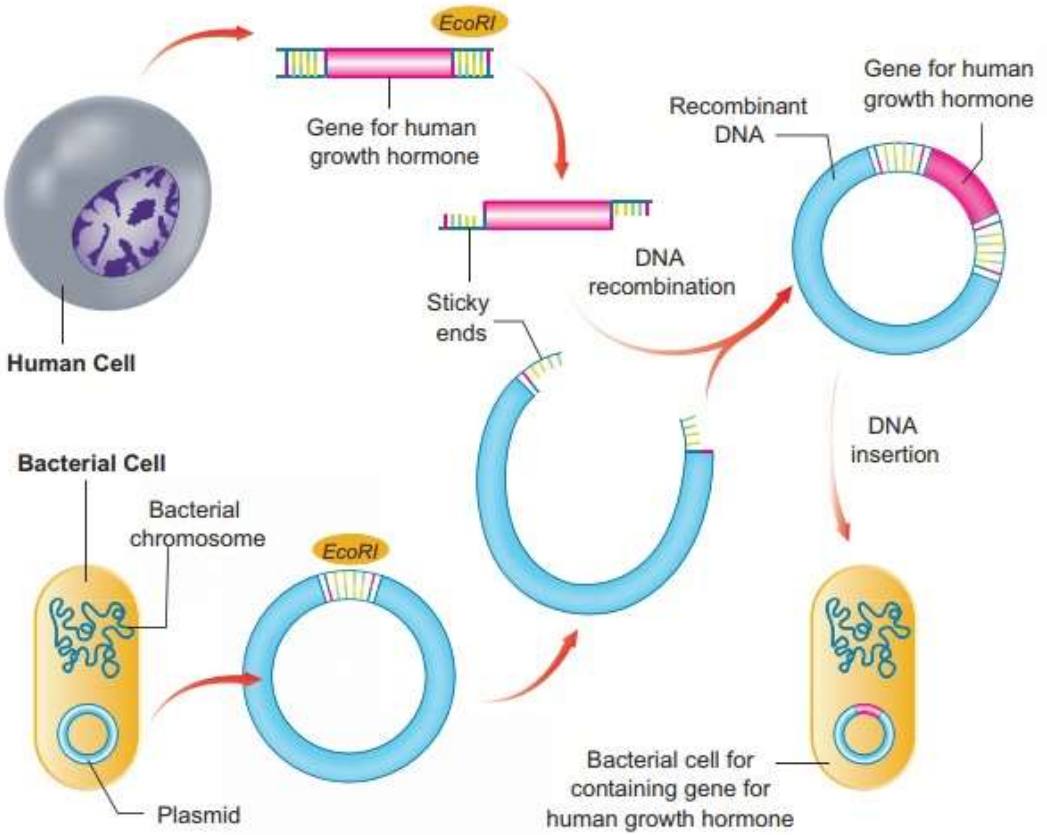
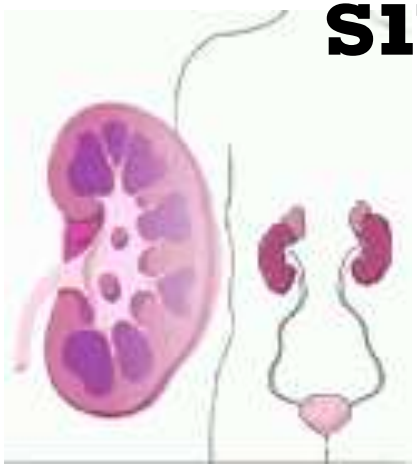



Fig. 10.2 Production of human growth hormone

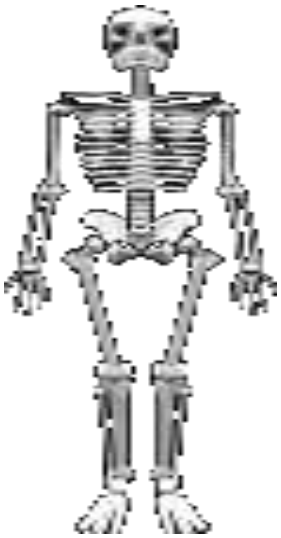
# ERYTHROPOIETIN

- Human Erythropoietin is produced in kidney
- A glycoprotein, acts on the bone marrow to increase the production of red and white blood cells. Stimuli such as bleeding or moving to high altitudes (where oxygen is scarce) trigger the release of erythropoietin
- Known as EPO, MW 30.4 KDa, 165 amino acids in human (192 Mouse)
- Has been widely used in AIDS for development of immunity

# Kidney is the principal production site of Erythropoietin



EPO

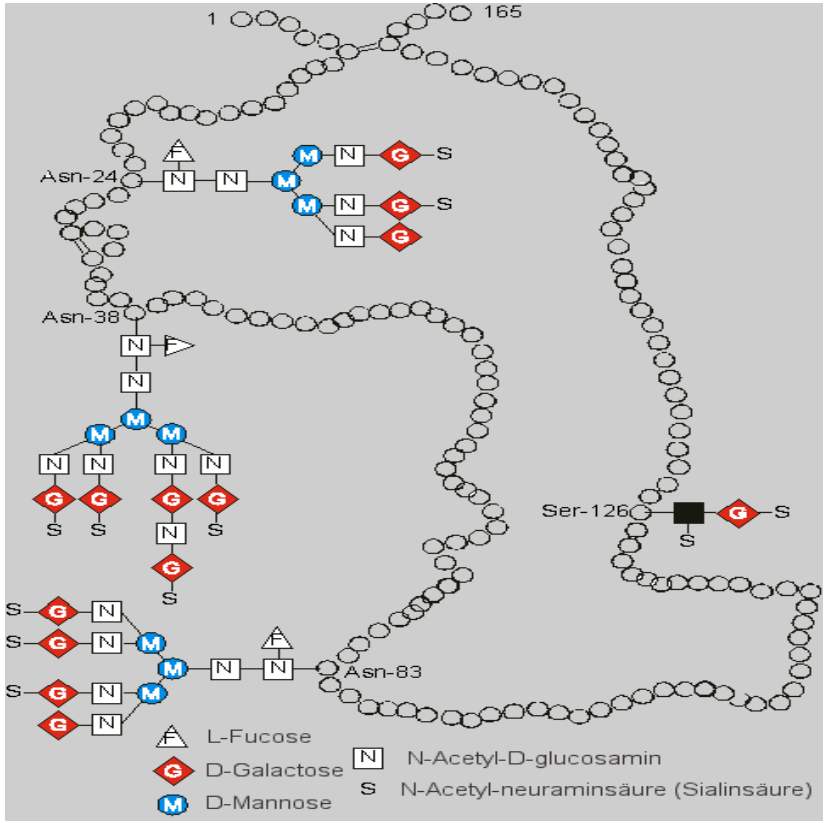


EPO is produced when RBC count comes down (Oxygen level decreases) in the blood

It will result in Kidney cells specifically sense the oxygen deficit in the blood and start producing Erythropoietin

Bones like Femur, Tibia, Vertebra, Sternum, Rib produce most RBC and WBC under the influence of EPO

# STRUCTURE OF EPO
# ( A GLYCOPROTEIN OF 165 AMINO ACIDS)




- Can be abused in sports to improve endurance
- Now detected from naturally occurring EPO by protein markers produced during post injection phase

# PRODUCTION OF RECOMBINANT ERYTHROPOIETIN

- Isolating and constructing human EPO cDNAs
- Subjecting the cDNA to PCR  using primers based on the published sequence
- The PCR products will be cloned into vector for the purpose of propagation and subsequently engineered into appropriate expression vectors

# Production process….

Genomic DNA, cDNA and manufactured DNA sequences coding for part or all the sequence of amino acid residues of EPO or for analogs thereof are incorporated into autonomously replicating plasmid or viral vectors employed to transform or transfect suitable prokaryotic or eukaryotic host cells such as bacteria, yeast or vertebrate cells in culture

Upon isolation from culture media or cellular lysates or fragments, products of expression of the DNA sequences display, e.g. the immunological properties and *in vitro* and *in vivo* biological activities of EPO of human or monkey species origins will be tested

# Flow Chart of Production Process

Vial (Cells) → Flask/ Spinner Bottle → Roller Bottle / Bio-reactor → Production → Purification → **Final Bulk EPO concentrate**

# CLOTTING FACTOR VIII

- The genes for the formation of factor VIII is located in the X chromosome.
- A genetic defect in the synthesis of factor VIII results in Haemophilia A, a sex-linked disease characterized by prolonged clotting time and internal bleeding.
- Clotting factor VIII isolated from blood of normal human being was used in the treatment of Haemophilia A. Requirement of large quantities of blood for this purpose and the risk of transmission of infectious diseases like AIDS is a disadvantage.

# CLOTTING FACTOR VIII

- Recombinant DNA technology was used to produce Recombinant Factor VIII in the Chinese Hamster ovary and in the baby Hamster kidney cells.
- More recently a cell line of human origin has been used for the first time to produce human blood clotting factor VIII.

# VACCINES

Vaccines are effective against many viral infections and diseases require the cultivation and mass production of the virus followed by its attenuation

The drawback in this is that virus requires a living medium to replicate and multiply. Rather than the traditional concept- "Sacrifice one life to save many", Animal cell culture can be employed to mass produce the virus

Passively, Animal cell culture can be employed to reduce the virulence of particular virus strains by cultivating them on cells other than target cells, in which the virus infection followed by repeated passaging will be performed

# VACCINES

The cell-culture process for vaccines offers high potential as an alternative method to egg-based production. Cell culture has the capability to offer a predictable, rapid and responsive method for production of well-tolerated and effective vaccines, with low levels of adverse events similar to egg-based vaccines

Cell-culture materials can be stored, so the production process can be initiated at any time. In addition, production can be scaled up in response to increased vaccine demand

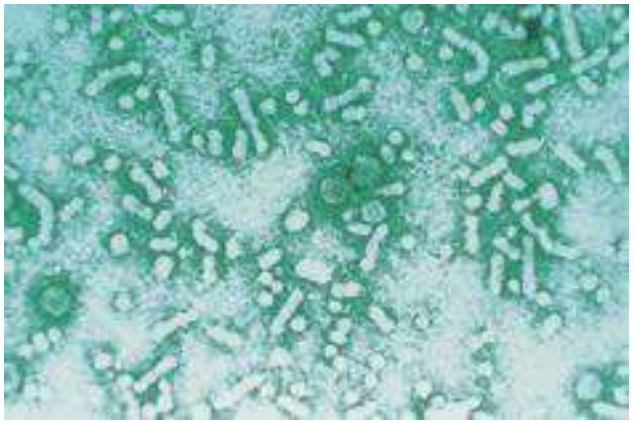
# PROBLEMS WITH THE EARLY TRADITIONAL VACCINE

- For many years the vaccine was produced by growing the live virus in animals and then inactivating it by chemical treatment

- This led to:
  - The risk of infection during the manufacture
  - and in the delivery of the vaccine
  - Raising animal rights issues

# RECOMBINANT HEPATITIS VACCINE

- The hepatitis B virus (HBV) vaccine
  - Originally based on the surface antigen purified from the blood of chronically infected individuals.
  - Due to safety concerns, the HBV vaccine became the first to be produced using recombinant DNA technology (1986)
  - Produced in bakers' yeast (Saccharomyces cerevisiae)



**Electron micrograph of the hepatitis B virus**

# RECOMBINANT HEPATITIS B VACCINE

- One of the most recent developments is the production of a vaccine against hepatitis B using genetically modified yeast cells

- Hepatitis B is a viral infection which attacks the cells of the liver. It can be very serious, causing chronic liver failure, liver cancer and death

- Hepatitis B can be prevented by a vaccination, and in where it is relatively common

# THE PROCESS

- Gene encoding the 226 amino acid hepatitis B surface antigen (HBsAg), was cloned into yeast
- The 5' end of the HBsAg gene was replaced with another DNA segment so as to optimize synthesis in yeast
- High-cell-density fermentations of laboratory strains of yeast have been developed for the production of HBsAg
- The HBsAg (lipoprotein particle) in cell lysates has been purified to obtain homogeneity

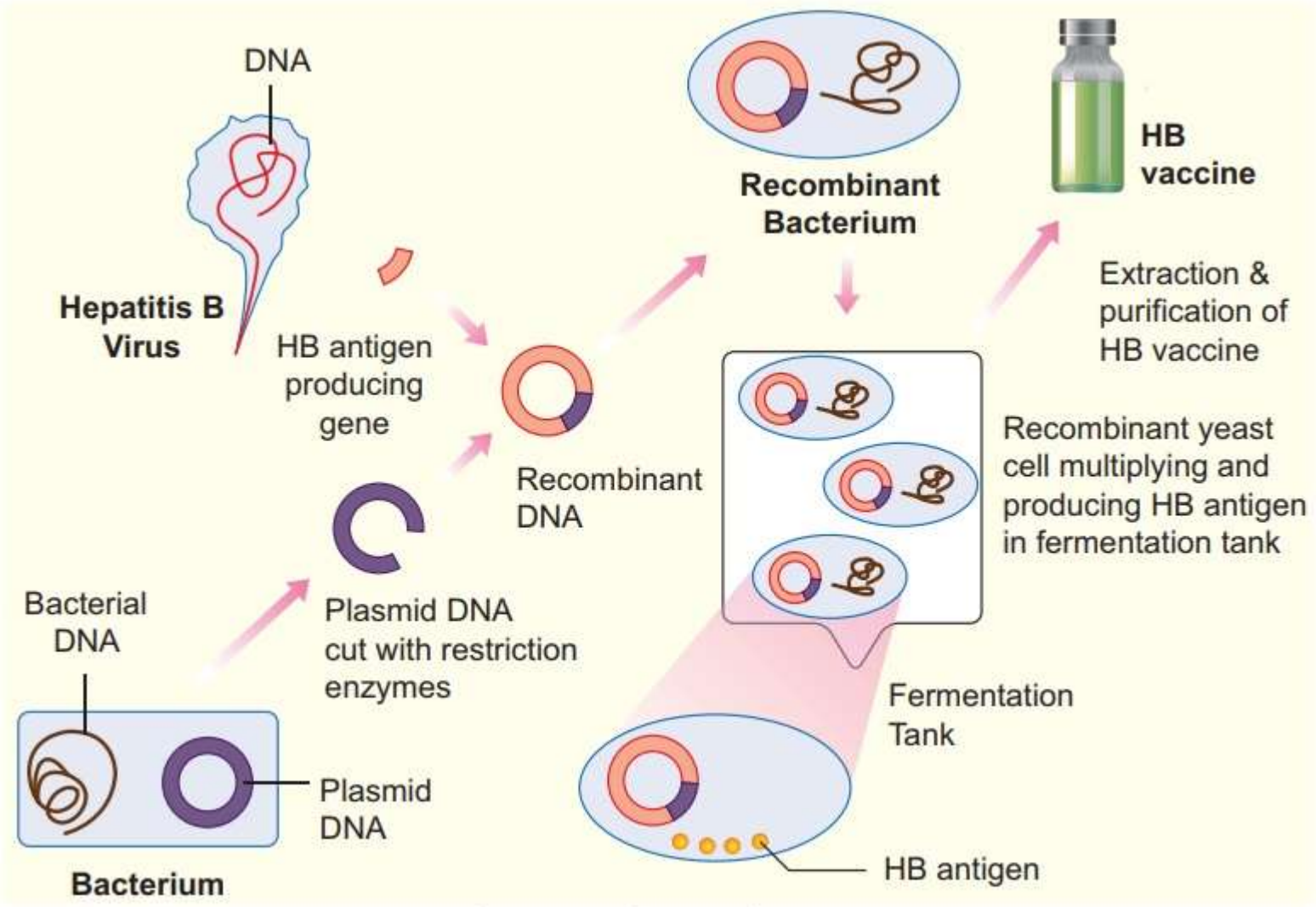



**Fig. 10.3 Production of recombinant HB Vaccine**

The recombinant vaccine for hepatitis B (HbsAg) was the first synthetic vaccine launched in 1997 which was marketed by trade names Recombivax and Engerix-B.

# Vaccine Production at industry level

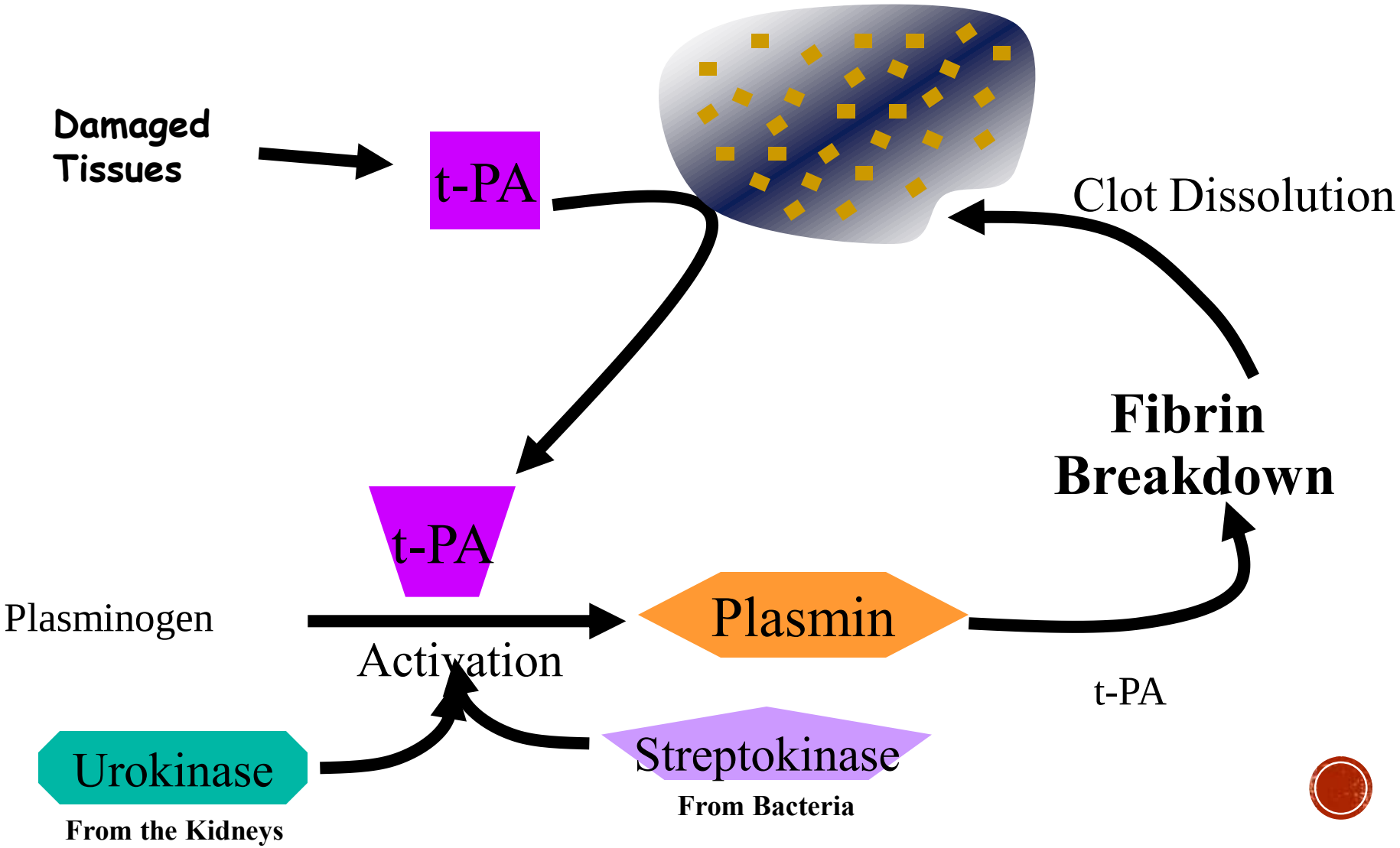
| Human vaccines | | Veterinary vaccines | |
|---|---|---|---|
| Virus | Cell used for culture | Virus | Cell used for culture |
| Measles | Chick embryo fibroblasts | Canine distemper | Chick embryo Fibroblasts or dog |
| Polio | Monkey kidney | Canine hepatitis | Dog |
| Polio (live) | Monkey kidney or human diploid cells | Foot and mouth disease | Bovine kidney cells |
| Rabies | Human diploid cells | Rabies | Duck embryo or Chick |
| Rubella | Rabbit kidney, duck embryo or human diploid cells | Feline panleucopenia | Cat kidney |
| | | | |
| | | Marek's disease | Chick embryo cells |

Some viral vaccines currently available for human and veterinary use

# TISSUE PLASMINOGEN ACTIVATOR

- The current generation of thrombolytic agents constitute t-PA, Urokinase, Streptokinase, Alteplase etc.
- Thrombolysins derived from eukaryotic cells (t-PA and Urokinase) can efficiently degrade the clots.
- But, they cannot meet the clinical requirements of the day because they pose greater problems in commercial production due to their large molecular size and expression incompatibilities

# TPA MODE OF ACTION



Major bottlenecks in clot dissolution by Therapeutics

- Reocclusion
- Half life
- Antigenicity
- Production cost

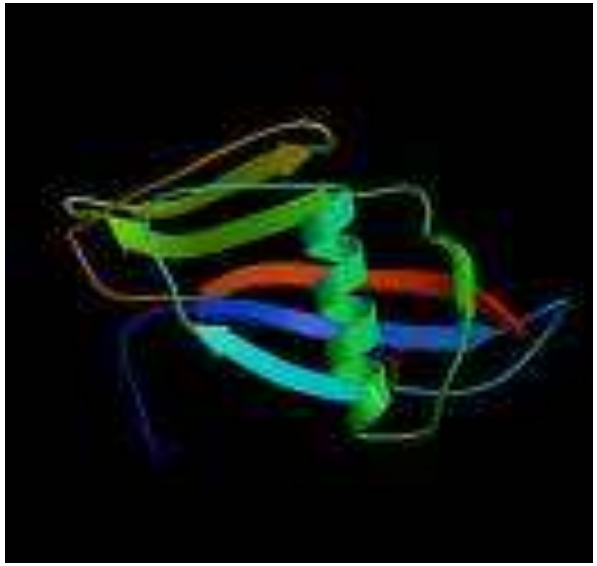
# Streptokinase

- Widely using thrombolytic agent
- Cheaper than all
- Not Clot specific, can lead to Systemic hemorrhage
- Even it is dangerous, it is very popular because of its low cost

•In contrast, the Staphylokinase has surpassed these incompatibilities and has proved to be a better alternative

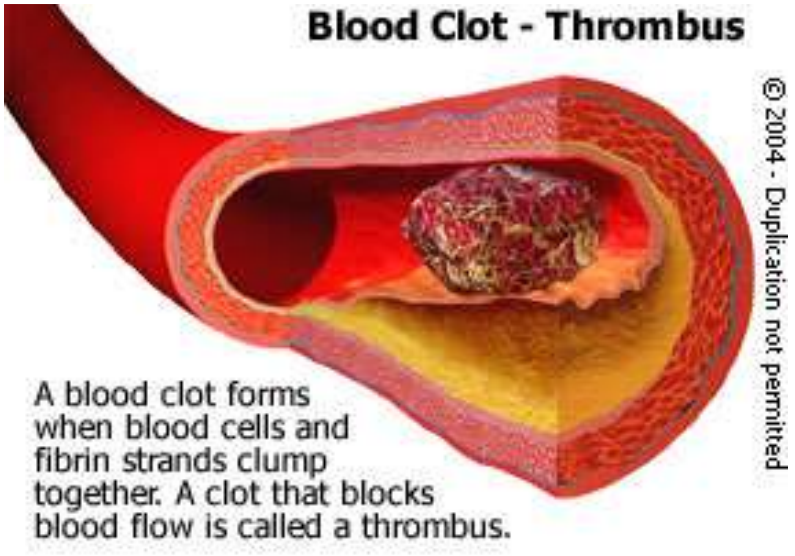
# Staphylokinase as a better alternative

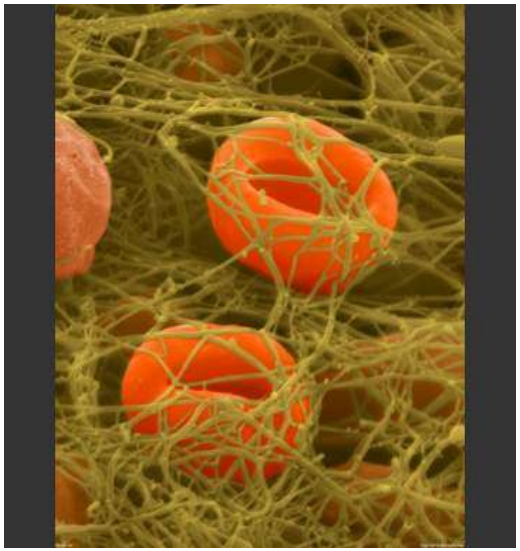
- Simple protein
- Small size
- Less antigenic than the streptokinase
- High Fibrin specificity
- New chimera with staphylokinase was developed (a patent was filed)



# STAPHYLOKINASE

- It is a upcoming 3rd generation thrombolytic agent. It can dissolve the blood clot effectively compared to the existing agents



# ALTEPLASE

- The biotechnology company Genentech has cloned human tissue Plasminogen Activator (Activase®) for use in treating unwanted or life threatening blood clots

- Activase is useful in treating heart attacks and strokes when administered within 5 hours of thrombosis formation or embolism lodging in the heart or brain
- Cathflo activase can be used to dissolve the clot blocking the catheter
- The FDA approval in 1987 and medical use of Activase has a very interesting history

- Alteplase is a glycoprotein of 527 amino acids. It is synthesized using the cDNA for natural human tissue-type plasminogen activator (t-PA) obtained from an established human cell line.

- The manufacturing process involves secretion of the enzyme Alteplase into the culture medium by Chinese hamster ovary cell lines into which the cDNA for Alteplase has been genetically inserted.

- Fermentation is carried out in a nutrient medium containing the antibiotic gentamicin sulfate, 100 mg/L. The presence of the antibiotic is not detectable in the final product.

# TUMOR NECROSIS FACTOR

- Tumor necrosis factor (TNFα) is a cytokine produced by immune cells having a capacity to suppress tumor cell proliferation and induce tumor regression.
- TNF is a protein consisting of 157 amino acids and is synthesized as a membrane-bound protein (pro-TNF) that is released by TNF-converting enzyme (TACE)-mediated cleavage.
- A variety of roles for TNF under physiological conditions were revealed such as in body development and immunity, and in pathological responses such as inflammation, tumor growth, transplant rejection, rheumatoid arthritis, and septic shock
- On the one hand, **its anticancer** property is mainly through inducing cancer cell death, a process that could be used for cancer therapy. On the other hand, TNF stimulates proliferation, survival, migration, and angiogenesis in most cancer cells that are resistant to TNF-induced cytotoxicity, resulting in tumor promotion.

# TUMOR NECROSIS FACTOR ALPHA

- While a number of studies have shown that tumor necrosis factor alpha (TNF-α) is a potent antitumor agent, it has not been widely used in this capacity because of its severe toxicity.
- If TNF-α could be delivered directly to its site of action, i.e., the tumor, then lower doses could be used and the unwanted side effects would be diminished.
- To develop a version of TNF-α with tumor specificity, DNA encoding the peptide Cys-Asn-Gly-Arg-Cys-Gly (which targets a tumor cell surface protein) was fused to TNF-α DNA.

# TUMOR NECROSIS FACTOR ALPHA

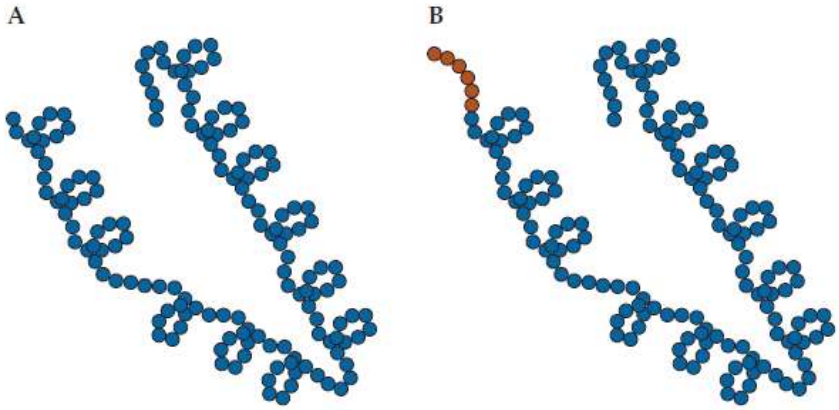
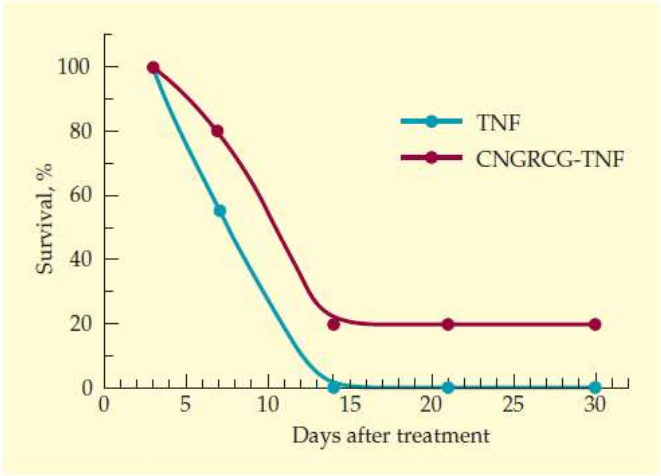



FIGURE 10.9 Schematic representation of TNF-α (blue) without (A) and with (B) a 6-amino-acid peptide (red) fused to its N terminus. The protein structure shown is hypothetical; only the numbers of amino acid residues (shown as circles) are accurately depicted.

FIGURE 10.10 Survival of lymphoma-bearing mice following treatment with 3 μg of either TNF-α or Cys-Asn-Gly-Arg-Cys-Gly–TNF-α (CNGRCG–TNF) as a function of the number of days after treatment.



# INTERFERONS

- Interferons are proteinaceous, antiviral, species specific substances produced by mammalian cells when infected with viruses.
- Based on the structure of interferons they are classified as $\alpha$, $\beta$ and $\gamma$ interferons.
- They stimulate the cellular DNA to produce antiviral enzymes which inhibit viral replication and protect the cells.
- Similar to factor VIII , interferons could be isolated from blood, but the amount of blood required for isolation of interferons is enormous and not practical.

# INTERFERONS

- To overcome this issue interferons could be produced by rDNA technology. The yeast *Saccharomyces cerevisiae* is more suitable for production of recombinant interferons than *E.coli*, since *E.coli* does not possess the machinery for glycosylation of proteins.
- Interferons are used for the treatment of various diseases like cancer, AIDS, multiple sclerosis, hepatitis C and herpes zoster.
- In spite of the therapeutic applications interferons are not within the reach of the common man due to high cost for its production

# INTERFERON ALFA-2B

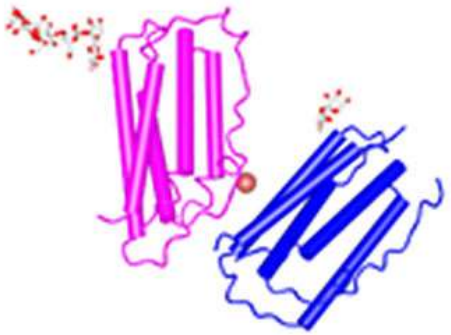
- Interferon alfa-2b is made from human proteins. **Interferons** help your body's immune system respond to bacteria, viruses, cancer, or other invading substances.
- Interferon alfa-2b is used to treat hairy cell **leukemia**, malignant melanoma, **follicular lymphoma**, **Kaposi's sarcoma** caused by AIDS, and certain types of **genital warts**.
- Interferon alfa-2b is also used to treat **chronic hepatitis B or C** in adults, and to treat chronic hepatitis B in children who are at least 1 year old

# INTERFERON ALFA-2B

- **Interferon alfa-2b should not be used together with ribavirin in pregnancy, or in a man whose wife is pregnant.** Pregnancy while using interferon alfa-2b should be prevented and for at least 6 months after stopping its use.
- It can cause life-threatening infections, **autoimmune disorders**, serious mood or behavior problems, or a stroke.

# INTERFERON BETA-1A

- Interferon beta-1a is used to treat relapsing **multiple sclerosis** (MS) in adults.
- interferon beta-1a will not cure MS, it will only decrease the frequency of relapse symptoms.
- Interferon beta-1a can **harm the liver** causing symptoms such as nausea, loss of appetite, tiredness, confusion, easy bruising, dark urine, clay-colored stools, or yellowing of skin or eyes.



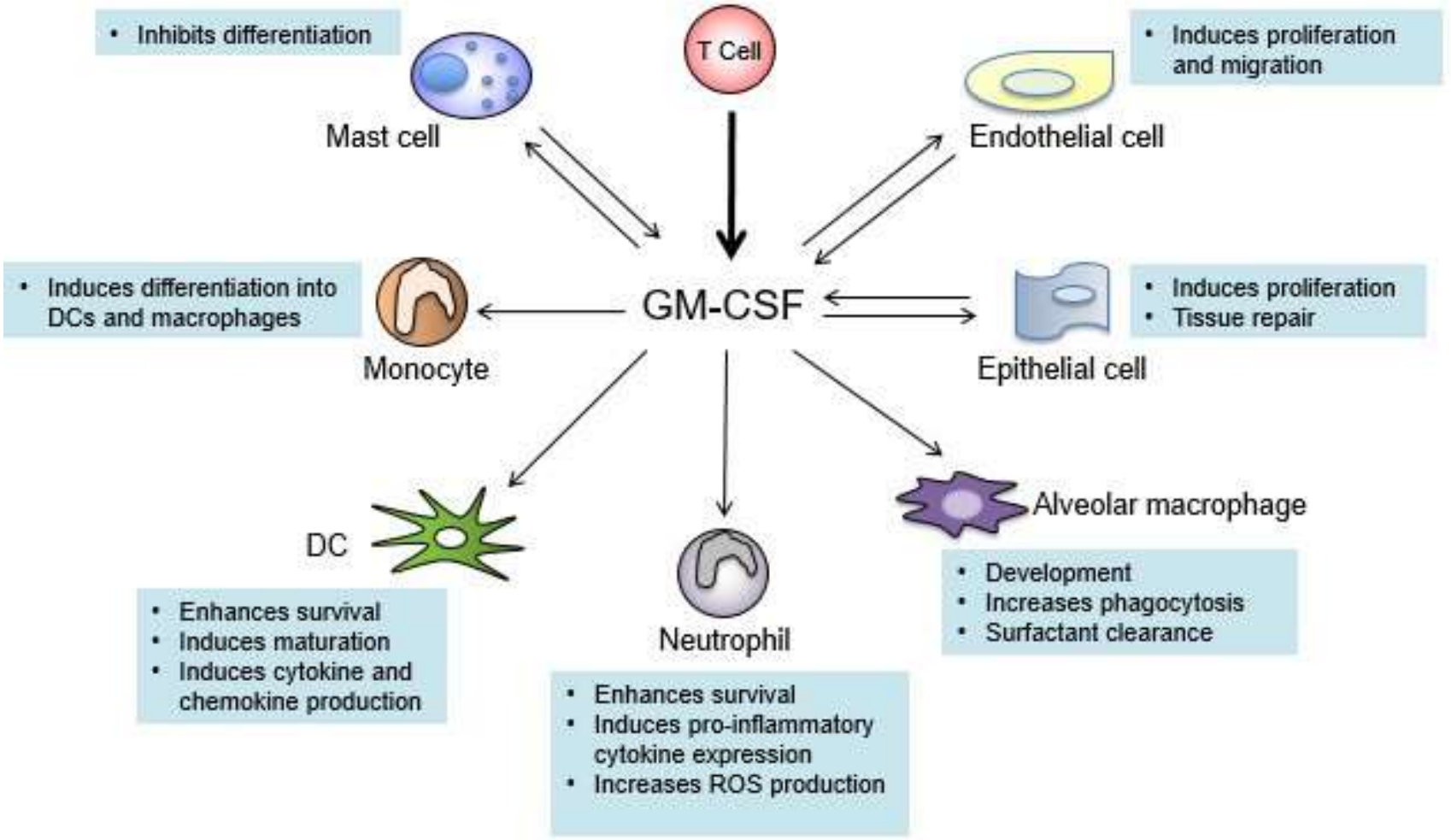
# INTERFERON GAMMA

- Interferon-γ (IFN-γ) is a cytokine that facilitates the amplification of the acute inflammatory response, particularly the stimulation of cytokine secretion, phagocytosis, and respiratory burst activity by macrophages
- Interferon-γ 1b is approved by the U.S. FDA to treat:
- **Chronic granulomatous disease** is a diverse group of hereditary diseases in which certain cells of the immune system have difficulty forming the reactive oxygen compounds (most importantly the superoxide radical due to defective phagocyte NADPH oxidase) used to kill certain ingested pathogens. This leads to the formation of granulomata in many organs.
- **Osteopetrosis** (Stone bone, inherited)
- Although not officially approved, Interferon-γ has also been shown to be effective in treating patients with **moderate to severe atopic dermatitis**

# GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)

- Filgrastim is used to stimulate the production of granulocytes in patients undergoing chemotherapy that will cause low white blood cell counts. This medication is used to prevent infection and neutropenic fevers caused by chemotherapy.
- Filgrastim is a support medication. It does not treat cancer
- There are different types of G-CSF, including:
  - lenograstim (Granocyte)
  - filgrastim (Neupogen, Zarzio, Nivestim, Ratiograstim)
  - long acting (pegylated) filgrastim (pegfilgrastim) and lipegfilgrastim (Lonquex)

# GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)



# GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)

- How it works:
- Before a stem cell transplant, G-CSF is used to stimulate the bone marrow to produce stem cells and release them into the blood. The stem cells are collected and then they administer high dose of chemotherapy.
- The high dose of chemotherapy stops the production of blood cells in bone marrow. So the stem cells back are injected into the bloodstream to start making the different types of blood cells again.
- Side effects include: bone pain, headache, liver changes, thrompocytopenia and diarrhea

# ENZYMES

- **DNAase:** used to lyse thick mucus in patients with cystic fibrosis having bacterial infection
- The thick mucus is rich in alginate produced by bacteria, DNA from dead bacteria and degenerating leukocytes
- To address this problem, scientists at the U.S. biotechnology company Genentech isolated the gene for the human enzyme deoxyribonuclease I (DNase I) and subsequently expressed the gene in Chinese hamster ovary (CHO) cells in culture.
- DNase I can hydrolyze long polymeric DNA chains into much shorter oligonucleotides.
- The purified enzyme was delivered in an aerosol mist to the lungs of patients with cystic fibrosis. The DNase I decreased the viscosity and adhesivity of the mucus in the lungs and made it easier for these patients to breathe

# ALGINATE LYASE AND BIOFILMS

- Alginate is a polysaccharide polymer that is produced by a wide range of seaweeds and both soil and marine bacteria.
- Alginate is composed of chains of the sugars β-d-mannuronate and α-l-guluronate.
- The cross-linked alginate polymer forms an elastic gel.
- The excretion of alginate by mucoid strains of *Pseudomonas aeruginosa* that infect the lungs of patients with cystic fibrosis significantly contributes to the viscosity of the mucus in the airways.
- Once mucoid strains of *P. aeruginosa* have become established in the lungs of cystic fibrosis patients, it is almost impossible to eliminate them by antibiotic treatment due to the formation of biofilms
- the addition of alginate lyase, which can liquefy bacterial alginate, together with or prior to antibiotic treatment, significantly decreased the number of bacteria found in biofilms

# ALGINATE LYASE AND BIOFILMS

- An alginate lyase gene has been isolated from a *Flavobacterium* species, a gram-negative soil bacterium that is a strong producer of this enzyme.
- A *Flavobacterium* clone bank was constructed in *E. coli* and screened for alginate lyase-producing clones by plating the entire clone bank onto solid medium containing alginate.
- Following growth, colonies that produced alginate lyase formed a halo around the colony when calcium was added to the plate
- In the presence of calcium, all of the alginate in the medium, except in the immediate vicinity of an alginate lyase-positive clone, becomes cross-linked and opaque. Since hydrolyzed alginate chains do not form cross-links, the medium surrounding an alginate lyase-positive clone is transparent.

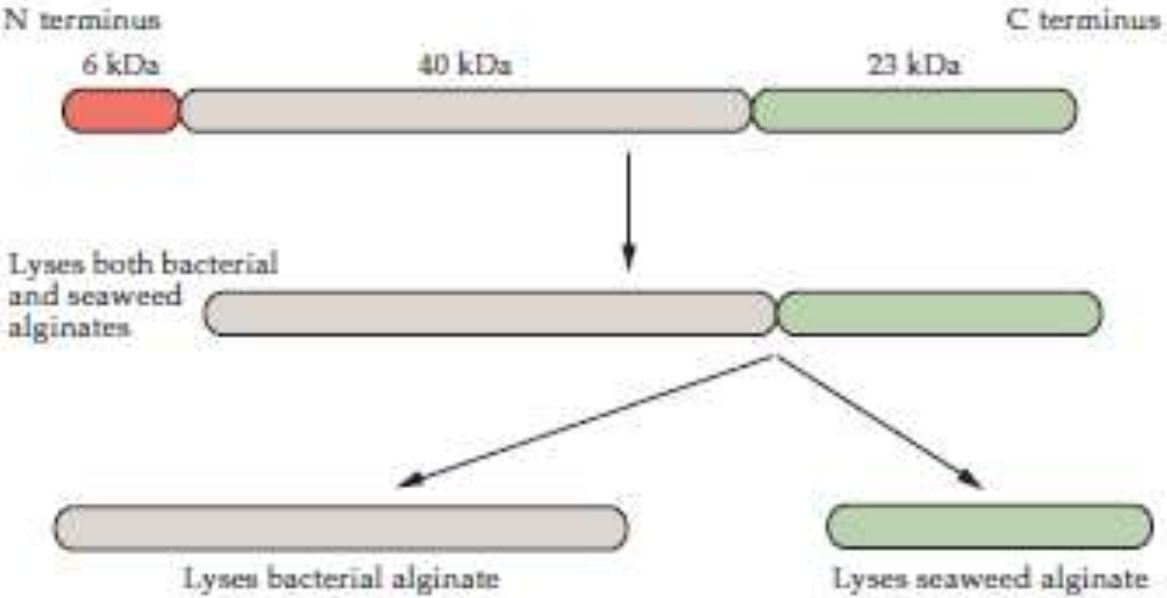
# ALGINATE LYASE AND BIOFILMS

- Analysis of a cloned DNA fragment from one of the positive colonies revealed an open reading frame encoding a polypeptide with a molecular mass of approximately 69,000 Da.
- After the 69,000-Da precursor is produced, a proteolytic enzyme cleaves off an N-terminal peptide of about 6,000 Da. The 63,000-Da protein can lyse both bacterial and seaweed alginates.
- Cleavage of the 63,000-Da protein yields a 23,000-Da enzyme that depolymerizes seaweed alginate and a 40,000-Da enzyme that is effective against bacterial alginate.
- To produce large amounts of the 40,000-Da enzyme, the DNA corresponding to the enzyme was amplified by the polymerase chain reaction (PCR) and then inserted into a *Bacillus subtilis* plasmid vector fused to a *B. subtilis* α-amylase leader peptide to direct the secretion of the protein and placed under the transcriptional control of a penicillinase gene promoter.

# ALGINATE LYASE AND BIOFILMS

- Transformation of *B. subtilis* cells with this construct yielded colonies with large halos on solid medium containing alginate after calcium was added. When these transformants were grown in liquid medium, the recombinant alginate lyase was secreted into the culture broth.
- Additional studies are necessary to determine whether recombinant alginate lyase is an effective therapeutic agent.

FIGURE 10.15 Processing of the recombinant *Flavobacterium* alginate lyase protein precursor in *E. coli*. A 6-kDa peptide is removed from the N terminus of the 69-kDa precursor to yield a 63-kDa protein that can depolymerize alginate from both seaweed and bacteria. A second cleavage event converts the 63-kDa protein into a 23-kDa protein that is active against seaweed alginate and a 40-kDa protein that hydrolyzes bacterial alginate.



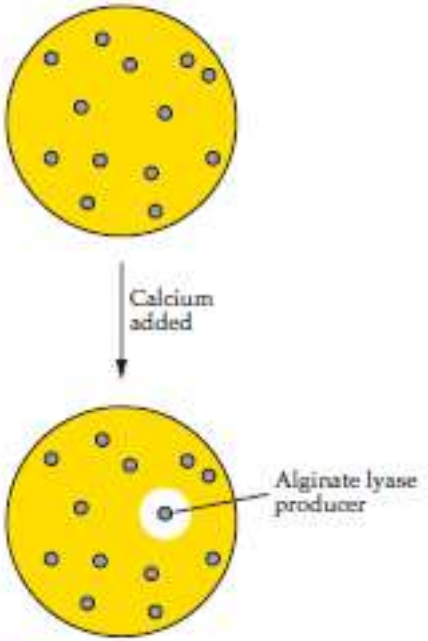




FIGURE 10.14 Schematic representation of the detection of an alginate lyase-producing clone from a clone bank of a *Flavobacterium* sp. in *E. coli*. The alginate that is present in the growth medium is digested by alginase secreted by an *E. coli* clone. The alginate in the vicinity of such a colony is not cross-linked when calcium is added and instead produces a clear zone (halo) surrounding the colony.

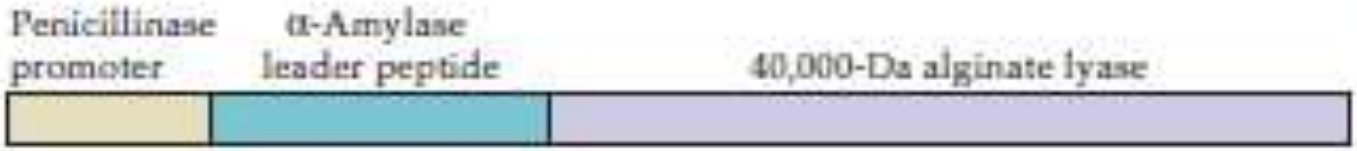



FIGURE 10.16 DNA construct encoding the 40,000-Da alginate lyase. The leader peptide from a *B. subtilis* α-amylase gene is fused to the N terminus of the alginate lyase coding sequence. The construct is under the transcriptional control of a *B. subtilis* penicillinase gene expression system.

# PHENYLALANINE AMMONIA LYASE

- The human genetic disease phenylketonuria results from the impaired functioning of the enzyme phenylalanine hydroxylase.
- When phenylalanine hydroxylase, which oxidizes phenylalanine to tyrosine, is deficient, the normal cognitive development of an individual is impaired and mental retardation ensues due to a buildup of phenylalanine.
- Following diagnosis of phenylketonuria, either prenatally or shortly after birth, the treatment entails a controlled semisynthetic diet with low levels of phenylalanine through infancy and possibly for life. A possible alternative treatment would be the administration of the enzyme phenylalanine hydroxylase.
- Unfortunately, phenylalanine hydroxylase is a multienzyme complex that is not very stable and requires a cofactor for activity while phenylalanine ammonia lyase, which converts phenylalanine to ammonia and *trans*-cinnamic acid, is a stable enzyme that does not require a cofactor and could potentially prevent the accumulation of phenylalanine in phenylketonuria patients

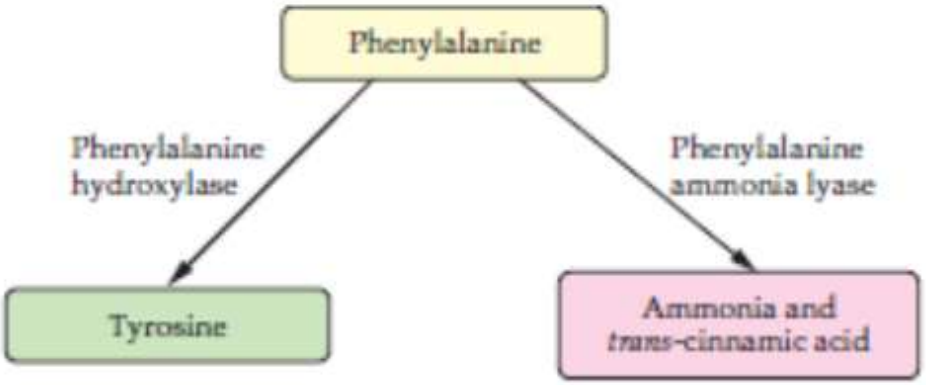
# PHENYLALANINE AMMONIA LYASE

- The gene for phenylalanine ammonia lyase from the yeast *Rhodosporidium toruloides* was cloned and overexpressed in *E. coli*.
- Preclinical studies were conducted with mice that were defective in producing phenylalanine ammonia lyase and therefore accumulated phenylalanine.
- With these mice, plasma phenylalanine levels were lowered when phenylalanine ammonia lyase was injected intravenously or encapsulated enzyme was administered orally.

# PHENYLALANINE AMMONIA LYASE

- phenylalanine ammonia lyase can be an effective substitute for phenylalanine hydroxylase, and the orally delivered enzyme is sufficiently stable to survive the mouse gastrointestinal tract and still function.

FIGURE 10.17 Products of the conversion of phenylalanine by phenylalanine hydroxylase and phenylalanine ammonia lyase.



# A1-ANTITRYPSIN

- The processing of a number of different pathogenic bacterial or viral precursor proteins by human proteases occurs when the protease recognizes the amino acid sequence –Arg-X-Lys/Arg-Arg↓–, with peptide bond cleavage on the C-terminal side of the C-terminal Arg, where X is any of the 20 common amino acids.
- Since this processing step is common to several infectious agents, a therapeutic agent that targeted the processing enzyme and blocked its activity might act as a broad-spectrum antibacterial and antiviral agent.
- A variant of human α1-antitrypsin was genetically engineered and tested in tissue culture experiments.

# A1-ANTITRYPSIN

- When the α1-antitrypsin variant was added to cell cultures, it blocked the production of human cytomegalovirus, a major cause of illness and death in organ transplant recipients and AIDS patients.
- The α1-antitrypsin variant is both potent and selective against human cytomegalovirus, it is at least 10-fold more effective than any currently used viral inhibitory agent.
- In addition to its use as replacement therapy in patients with deficiency

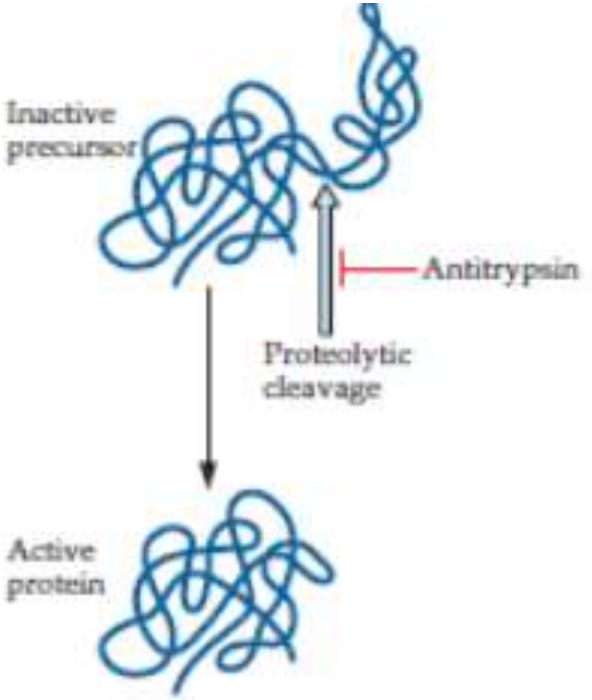



FIGURE 10.18 Schematic representation of $\alpha_1$-antitrypsin inhibiting the proteolytic cleavage of pathogenic precursor proteins by human proteases.

# BIOPOLYMERS

- Biopolymers are large, multiunit macromolecules synthesized by microorganisms, plants, and animals.
- Some of these polymers have physical and chemical properties that are useful to the food-processing, manufacturing, and pharmaceutical industries.
- The ability to genetically engineer organisms has stimulated researchers to:
  - Design new biopolymers
  - Replace synthetic polymers with biological equivalents
  - Modify existing biopolymers to enhance their physical and structural characteristics
  - Find ways to increase the yields and decrease the costs of biopolymers produced by industrial processes.

# XANTHAN GUM

- ***Xanthomonas campestris*** is a gram-negative obligatory aerobic **soil** bacterium that produces the commercially important biopolymer xanthan gum a high-molecular-weight exopolysaccharide, as a by-product of its metabolism.
- This polymer has a cellulosic backbone made up of a straight-chain polymer of glucose units. Each of its trisaccharide side chains includes one glucuronic acid and two mannose residues, which are attached to every second glucose residue of the backbone.
- Xanthan gum has **high viscosity, is stable** in extreme physical and chemical environments, and exhibits physical and chemical properties similar to those of a plastic.
- Its physical properties make it useful as a **stabilizing, emulsifying, thickening, or suspending agent**.

# XANTHAN GUM

- For successful commercial production of xanthan gum, *X. campestris* should be grown on an inexpensive and plentiful carbon source.
- Wild-type *X. campestris* can efficiently utilize glucose, sucrose, and starch, but **not lactose**, as a carbon source
- Whey is a waste by-product of the cheese-making process that consists of water (94 to 95%), lactose (3.5 to 4%), and small amounts of protein, minerals, and low-molecular-weight organic compounds.
- Enormous quantities of whey are generated by the dairy industry, and its disposal is a major problem.

# XANTHAN GUM

- In North America, whey has been used extensively as a **"filler"** in the preparation of prepared foods; however, with the increasing awareness that large numbers of individuals are **lactose intolerant**, it is imperative that alternative uses be found for this material.
- Disposing of whey by **releasing it into rivers and lakes** can deplete the amount of available oxygen, thereby killing many of the aquatic organisms. Transporting whey to landfill sites is **expensive**, and potential **groundwater contamination** by the discarded whey is a major concern.
- Finally, **the costs** of removing the solid component of whey are prohibitive so, use whey in creative ways.
- Theoretically, whey could be used as a carbon source for growing industrially important microorganisms. With this in mind, *X. campestris* was genetically engineered to grow on whey. The *E. coli lacZY* genes, which encode the enzymes β-galactosidase and lactose permease, were **cloned onto a broad-host-range plasmid under the transcriptional control of an *X. campestris* bacteriophage promoter.**

# XANTHAN GUM

- This construct was introduced into *E. coli* and then transferred from *E. coli* to *X. campestris.*
- Transformants maintained the plasmid, expressed the enzymes β-galactosidase and lactose permease at high levels, **utilized lactose** as the sole carbon source, and produced large amounts of xanthan gum with glucose, lactose, or whey as the carbon source.
- By contrast, wild type *X. campestris* produces large amounts of xanthan gum only when grown on glucose. This system may be able to convert a waste product into a substrate for the production of an economically valuable biopolymer.

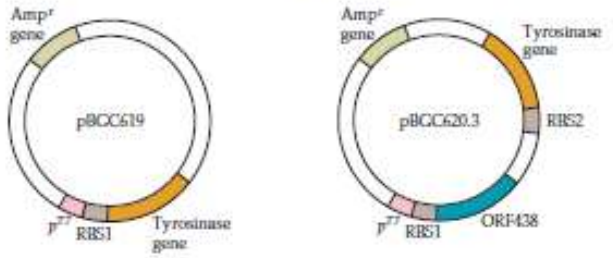
# MELANIN

- Melanins are a large, diverse family of light-absorbing biopolymers that are synthesized by animals, plants, bacteria, and fungi. It has been suggested that these pigments might be useful as **topical sunscreens, sunlight-protective coatings for plastics, or additives for cosmetic products**.
- Currently, melanins are obtained in small quantities either by extraction from natural sources or by chemical synthesis. However, recombinant DNA technology has made it possible to produce a **range of melanins** with different physical properties inexpensively and on a large scale.
- Biochemically, melanins are irregular, somewhat random polymers that are composed of indoles, benzthiazoles, and amino acids. The first step in their synthesis, which is catalyzed by the copper-containing monooxygenase **tyrosinase**, is the oxidation of tyrosine to dihydroxyphenylalanine quinone. The final stages of the polymerization of melanin are nonenzymatic, and depending on the chemical nature of the nonquinone components that are incorporated into the polymeric structure (typically hydroxylated organic compounds), the **end product can be black, brown, yellow, red, or violet**.

# MELANIN

- The genes involved in melanin biosynthesis in the bacterium *Streptomyces antibioticus* have been isolated and analyzed.
- Apotyrosinases are activated by acquiring copper ions.
- Under natural conditions, after  dihydroxyphenylalanine quinone is produced by tyrosinase, a variety of low-molecular-weight compounds (nonquinones) can be incorporated into the final polymer.
- The chemical and physical nature of the melanin that is formed after cloning of the key genes into *E. coli* may be manipulated to some extent, to form melanins with different properties, by the addition of different amounts of specific low-molecular-weight compounds to the medium.

FIGURE 13.33 *E. coli* expression plasmids carrying melanin biosynthesis genes. Plasmid pBGC619 contains the tyrosinase gene. Plasmid pBGC620.3 contains an ORF (ORF438) for melanin synthesis and the tyrosinase gene. Transcription of the cloned genes is under the control of the *E. coli* bacteriophage T7 promoter ($p^{T7}$). RBS1 and RBS2 denote two different ribosome-binding sites. The plasmids both carry genes that confer resistance to ampicillin ($Amp^r$).



# FOOD ADDITIVE - ASPARTAME

- Aspartame (L-phenylalanyl-L-aspartyl-methyl ester) is a low-calorie artificial sweetener

- It can be synthesised biocatalytically by peptide synthesis using a thermostable protease – Thermolysin® from the facultative thermophile, *Bacillus thermoproteolyticus*

# VALUE ADDED PRODUCTS

- **Acrylamide** is one of the most important chemical commodities, being in great demand (200 000 tons per year worldwide) as a starting material for the production of various polymers.

- **Nicotinamide** is a Vitamin that can also be synthesized in the same biological process

# USES

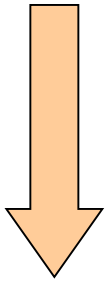
- Acrylamide
  - Absorbent polymers
  - Flocculants
  - Construction material

- Nicotinic acid
  - Animal feed supplement
  - Human Health food supplement

# PRODUCTION OF NICOTINAMIDE

- Nicotinamide is an essential vitamin, and is widely used in the health-food and animal food-and-feed industries. Biological production, using the same *Rhodococcus* biocatalyst as for acrylamide production, operates at about 5kT p.a.

3-cyanopyridine



*Rhodococcus* whole cell biocatalyst

Nicotinamide

# PRODUCTION OF ALKALOIDS FROM PLANTS

Effects of alkaloids on humans

- High biological activity
- Produce varying degrees of physiological and psychological responses - largely by interfering with neurotransmitters
  - others interfere with membrane transport, protein synthesis or other processes
- In large doses - highly toxic - fatal
- In small doses, many have therapeutic value
  - muscle relaxants, tranquilizers, pain killers, mind altering drugs, chemotherapy

| TABLE 9.1 | Some Pharmaceutical Products of Genetic Engineering |
|---|---|
| **Product** | **Comments** |
| Alpha-interferon | Therapy for leukemia, melanoma, and hepatitis; produced by *E. coli* and *Saccharomyces cerevisiae* (yeast). |
| Antitrypsin | Assists emphysema patients; produced by genetically modified sheep. |
| Beta-interferon | Treatment for multiple sclerosis; produced by mammalian cell culture. |
| Bone morphogenic proteins | Induces new bone formation; useful in healing fractures and reconstructive surgery; produced by mammalian cell culture. |
| Colony-stimulating factor (CSF) | Counteracts effects of chemotherapy; improves resistance to infectious disease such as AIDS; treatment of leukemia; produced by *E. coli* and *S. cerevisiae*. |
| Epidermal growth factor (EGF) | Heals wounds, burns, ulcers; produced by *E. coli*. |
| Erythropoietin (EPO) | Treatment of anemia; produced by mammalian cell culture. |
| Factor VIII | Treatment of hemophilia; improves clotting; produced by mammalian cell culture. |
| Gamma-interferon | Treatment of chronic granulomatous disease; produced by *E. coli*. |
| Hepatitis B vaccine | Produced by *S. cerevisiae* that carries hepatitis-virus gene on a plasmid. |
| Human growth hormone (hGH) | Corrects growth deficiencies in children; produced by *E. coli*. |
| Human insulin | Therapy for diabetes; better tolerated than insulin extracted from animals; produced by *Escherichia coli*. |