

Production of Protein Pharmaceuticals (Part 1)

Dr. David Wishart
Athabasca Hall 3-41
david.wishart@ualberta.ca

Protein Pharmaceuticals

- Actimmune (If γ)
- Activase (TPA)
- BeneFix (F IX)
- Betaseron (If β)
- Humulin
- Novolin
- Pegademase (AD)
- Epogen
- Regranex (PDGF)
- Novoseven (F VIIa)
- Intron-A
- Neupogen
- Pulmozyme
- Infergen

Today's lecture notes are available at:

<http://redpoll.pharmacy.ualberta.ca>

Protein Pharmaceuticals

- First "protein vaccine" was cow-pox (Jenner, 1796)
- First protein pharmaceutical was insulin (Banting & Best, 1922)
- Now more than 200 approved peptide and protein pharmaceuticals on the FDA list

(http://www.accessexcellence.org/RC/AB/IWT/The_Biopharmaceuticals.html)

- Many different sources...

Protein Pharmaceuticals



- Insulin (diabetes)
- Interferon β (relapsing MS)
- Interferon γ (granulomatous)
- TPA (heart attack)

Protein Pharmaceuticals

Protein Drug

Original Source

- Insulin Pigs or cattle (pancreas)
- Albumin Human blood (donated)
- HGH Human brains
- Factor VIII Human blood (donated)
- Calcitonin Salmon
- Anti-venom Horse of Goat blood

Protein Pharmaceuticals

- Natural sources are often rare and expensive
 - Difficult to keep up with demand
 - Hard to isolate product
 - Lead to immune reactions (diff. species)
 - Viral & pathogen contamination
- Most protein pharmaceuticals today are produced recombinantly
 - Cheaper, safer, abundant supply

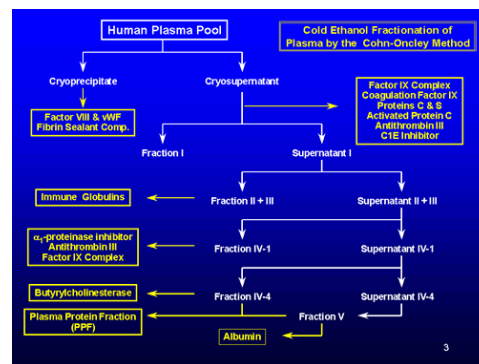
Key Blood Products

- Factor VIII (for blood clotting-hemophilia)
- Factor IX (for blood clotting-hemophilia)
- Albumin (osmotic balance-kidney disease)
- Ig IV (for treating infections)
- Anti-thrombin III (for blood clotting)
- Alpha I-PI (for emphysema, AIAD)
- All prepared by Cohn Fractionation (1946)
- Differential precipitation by ethanol, salt, pH, temperature, centrifugation

Protein Pharmaceuticals From Blood

- Body contains 6 litres of blood
- 60-70% of blood is plasma, 8-9% is protein – a **pharmaceutical cornucopia**
- Plasma contains 10,000 different proteins (~20 proteins make up 99% of plasma proteins)
- Discarded, donated blood ~2 million litres/year – **great source for proteins**

Cohn Fractionation



Blood Fractionation



Peptide Drugs

- Many hormones are actually small peptides (2-40 amino acids)
- Calcitonin (Calcimar, Miacalcin, 32 res.)
 - Thyroid hormone to enhance bone mass
- Oxytocin (Pitocin, 9 residues)
 - Pituitary hormone to stimulate labor
- Vasopressin (Pitressin, 9 residues)
 - Pituitary h. for antidiuretic/vasoconstriction

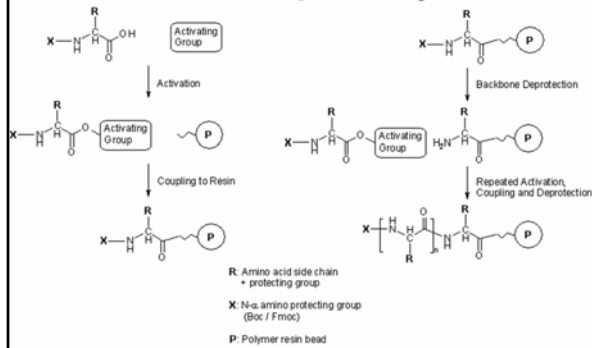
Peptide Drugs

- Small enough to synthesize using solid phase chemistry (SPPS)
- Method developed by Bruce Merrifield in 1960's (won Nobel prize)
- Very efficient synthesis (>99%/couple)
- Two different chemical approaches
 - Boc (t-butoxycarbonyl - acid labile protecting group)
 - Fmoc (fluorenylmethoxycarbonyl - base labile protecting group)

Automatic Peptide Synthesizer (ABI 433A)



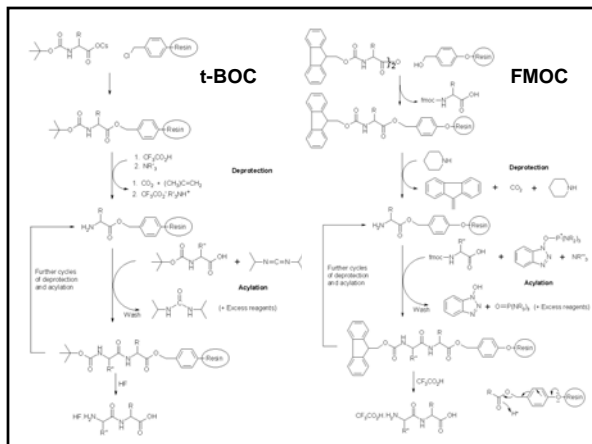
Solid Phase Peptide Synthesis



Peptide Coupling Efficiency

- 10 residue peptide, 95% coupling
 - Yield = $0.95^{10} = 59.8\%$
- 10 residue peptide, 99% coupling
 - Yield = $0.99^{10} = 90.4\%$
- 50 residue peptide, 99% coupling
 - Yield = $0.99^{50} = 60.5\%$

Still limited to small proteins/peptides



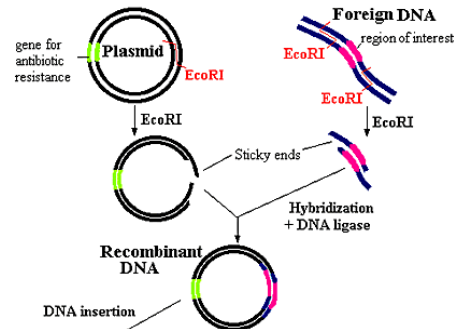
Protein Pharmaceuticals

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Recombinant Methods

- Developed in 1970's & 1980's
- Paul Berg (1973) restriction enzymes
- Herbert Boyer (1978) cloning human insulin into E. coli – Genentech
- Two general approaches
 - Expression in isolated cells
 - Expression in transgenic plants/animals

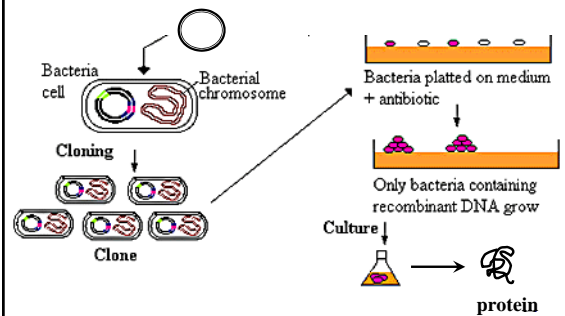
Cloning (Details)



Six Step Process

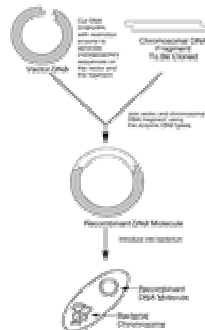
- Isolation of gene of interest
- Introduction of gene to expression vector
- Transformation into host cells
- Growth of cells through fermentation
- Isolation & purification of protein
- Formulation of protein product

Cloning (Details)



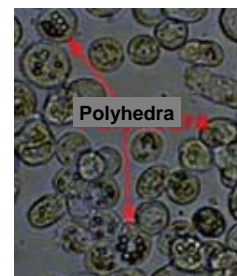
Cloning Process

- Gene of interest is cut out with restriction enzymes (RE)
- Host plasmid (circular chromosome) is cut with same REs
- Gene is inserted into plasmid and ligated with ligase
- New (engineered) plasmid inserted into bacterium (transform)



Recombinant Protein Expression Systems

- Escherichia coli
- Other bacteria
- Pichia pastoris
- Other yeast
- Baculovirus
- Animal cell culture
- Plants
- Sheep/cows/humans
- Cell free



Expression System Selection

- Choice depends on size and character of protein
 - Large proteins (>100 kD)? Choose eukaryote
 - Small proteins (<30 kD)? Choose prokaryote
 - Glycosylation essential? Choose baculovirus or mammalian cell culture
 - High yields, low cost? Choose E. coli
 - Post-translational modifications essential? Choose yeast, baculovirus or other eukaryote

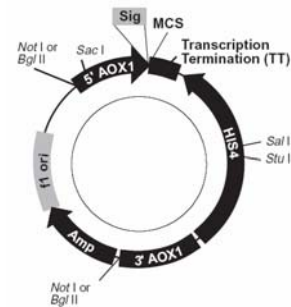
Key Parts to a Vector

- Origin of replication (ORI) – DNA sequence for DNA polymerase to replicate the plasmid
- Selectable marker (Amp or Tet) – a gene, when expressed on plasmid will allow host cells to survive
- Inducible promoter – Short DNA sequence which enhances expression of adjacent gene
- Multi-cloning site (MCS) – Short DNA sequence that contains many restriction enzyme sites

Which Vector?

- Must be compatible with host cell system (prokaryotic vectors for prokaryotic cells, eukaryotic vectors for eukaryotic cells)
- Needs a good combination of
 - strong promoters
 - ribosome binding sites
 - termination sequences
 - affinity tag or solubilization sequences
 - multi-enzyme restriction site

A Generic Vector



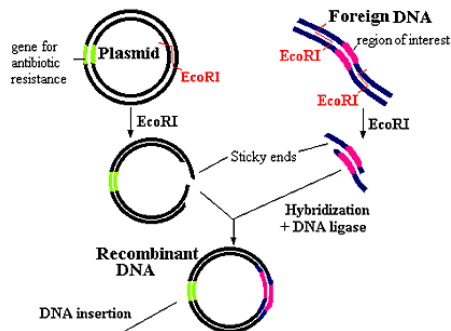
Plasmids and Vectors

- Circular pieces of DNA ranging in size from 1000 to 10,000 bases
- Able to independently replicate and typically code for 1-10 genes
- Often derived from bacterial “mini” chromosomes (used in bacterial sex)
- May exist as single copies or dozens of copies (often used to transfer antibiotic resistance)

Which Vector?

- Promoters
 - arabinose systems (pBAD), phage T7 (pET), Trc/Tac promoters, phage lambda PL or PR
- Tags
 - His₆ for metal affinity chromatography (Ni)
 - FLAG epitope tag **DYKDDDDK**
 - CBP-calmodulin binding peptide (26 residues)
 - E-coil/K-coil tags (poly E₃₅ or poly K₃₅)
 - c-myc epitope tag **EQKLISEEDL**
 - Glutathione-S-transferase (GST) tags
 - Cellulose binding domain (CBD) tags

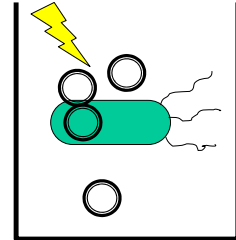
Gene Introduction (Bacteria)



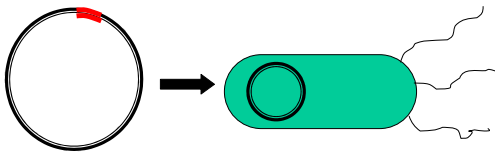
Electroporator



25 microfarads = 2500 V
@ 200 ohms for 5 ms

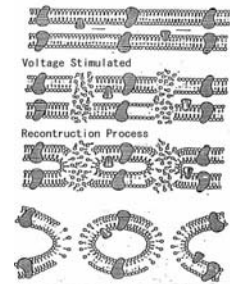


Bacterial Transformation



Electroporation

- Seems to cause disruption in cell membrane
- Reconstitution of membrane leads to large pores which allow DNA molecules to enter
- Works for bacteria, yeast and animal cells



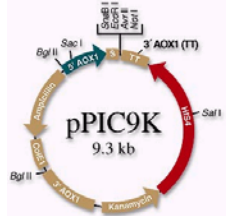
Bacterial Transformation

- Moves the plasmid into bacterial host
- Essential to making the gene “actively” express the protein inside the cell
- 2 routes of transformation
 - CaCl₂ + cold shock
 - Electroporation
- Typical transformation rate is 1 in 10,000 cells (not very efficient) for CaCl₂, but 1 in 100 for electroporation

Bacterial Systems

- | <u>Advantages</u> | <u>Disadvantages</u> |
|---|---|
| • Grow quickly (8 hrs to produce protein) | • Difficulty expressing large proteins (>50 kD) |
| • High yields (50-500 mg/L) | • No glycosylation or signal peptide removal |
| • Low cost of media (simple media constituents) | • Eukaryotic proteins are sometimes toxic |
| • Low fermentor costs | • Can't handle S-S rich proteins |

Cloning & Transforming in Yeast Cells



Pichia pastoris

Pichia Pastoris Cloning

- Uses a special plasmid that works both in *E. coli* and Yeast
- Once gene of interest is inserted into this plasmid, it must be linearized (cut open so it isn't circular)
- Double cross-over recombination event occurs to cause the gene of interest to insert directly into *P. pastoris* chromosome where the old AOX gene used to be
- Now gene of interest is under control of the powerful AOX promoter

Pichia Pastoris

- Yeast are single celled eukaryotes
- Behave like bacteria, but have key advantages of eukaryotes
- *P. pastoris* is a methylotrophic yeast that can use methanol as its sole carbon source (using alcohol oxidase)
- Has a very strong promoter for the alcohol oxidase (AOX) gene (~30% of protein produced when induced)

Pichia Systems

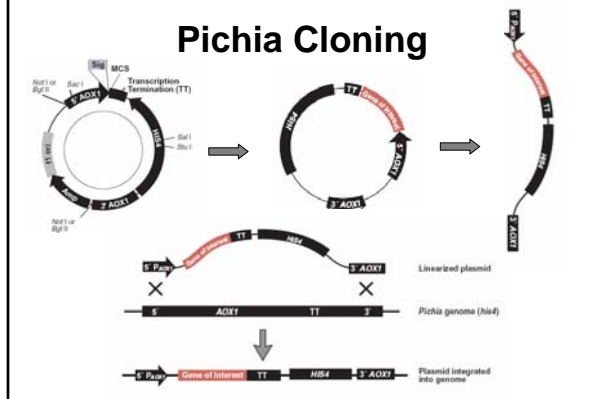
Advantages

- Grow quickly (8 hrs to produce protein)
- Very high yields (50-5000 mg/L)
- Low cost of media (simple media constituents)
- Low fermentor costs

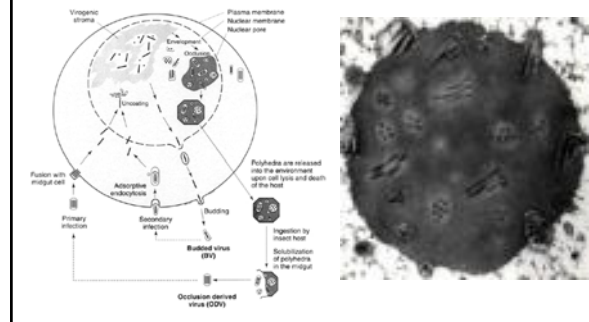
More advantages

- Can express large proteins (>50 kD)
- Glycosylation & signal peptide removal
- Has chaperonins to help fold "tough" prtns
- Can handle S-S rich proteins

Pichia Cloning



Baculovirus Expression



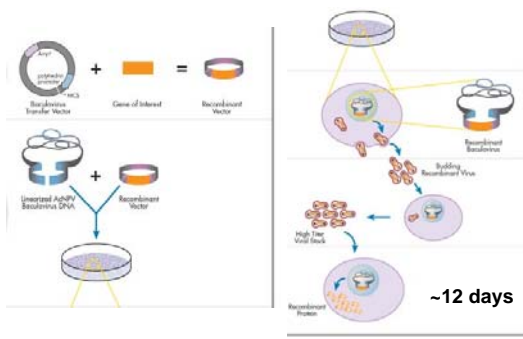
Baculovirus Expression

- *Autographica californica* multiple nuclear polyhedrosis virus (Baculovirus)
- Virus commonly infects insects cells of the alfalfa looper (small beetle) or armyworms (and their larvae)
- Uses super-strong promoter from the polyhedron coat protein to enhance expression of proteins while virus resides inside the insect cell

Baculovirus Successes

- Alpha and beta interferon
- Adenosine deaminase
- Erythropoietin
- Interleukin 2
- Poliovirus proteins
- Tissue plasminogen activator (TPA)

Baculovirus Expression



Baculovirus Systems

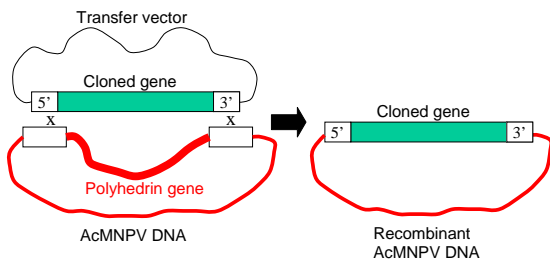
Disadvantages

- Grow very slowly (10-12 days for set-up)
- Cell culture is only sustainable for 4-5 days
- Set-up is time consuming, not as simple as yeast

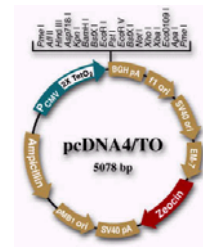
Advantages

- Can express large proteins (>50 kD)
- Correct glycosylation & signal peptide removal
- Has chaperonins to help fold "tough" prtns
- Very high yields, cheap

Baculovirus (AcMNPV) Cloning Process



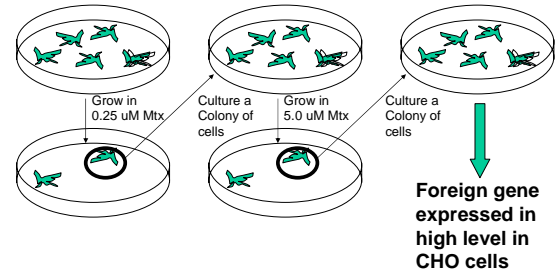
Mammalian Expression Systems



Mammalian Cell-line Expression

- Sometimes required for difficult-to-express proteins or for “complete authenticity” (matching glycosylation and sequence)
- Cells are typically derived from the Chinese Hamster Ovary (CHO) cell line
- Vectors usually use SV-40 virus, CMV or vaccinia virus promoters and DHFR (dihydrofolate reductase) as the selectable marker gene

Methotrexate (MTX) Selection



Mammalian Expression

- Gene initially cloned and plasmid propagated in bacterial cells
- Mammalian cells transformed by electroporation (with linear plasmid) and gene integrates (1 or more times) into random locations within different CHO chromosomes
- Multiple rounds of growth and selection using methotrexate to select for those cells with highest expression & integration of DHFR and the gene of interest

Mammalian Systems

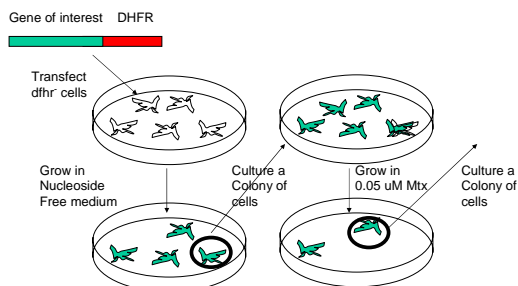
Disadvantages

- Selection takes time (weeks for set-up)
- Cell culture is only sustainable for limited period of time
- Set-up is very time consuming, costly, modest yields

Advantages

- Can express large proteins (>50 kD)
- Correct glycosylation & signal peptide removal, generates authentic proteins
- Has chaperonins to help fold “tough” prtns

Methotrexate (MTX) Selection



Mammalian Cell Successes

- Factor IX
- Factor VIII
- Gamma interferon
- Interleukin 2
- Human growth hormone
- Tissue plasminogen activator (TPA)

Conclusion

- Isolation of gene of interest
- Introduction of gene to expression vector
- Transformation into host cells
- Growth of cells through fermentation
- Isolation & purification of protein
- Formulation of protein product