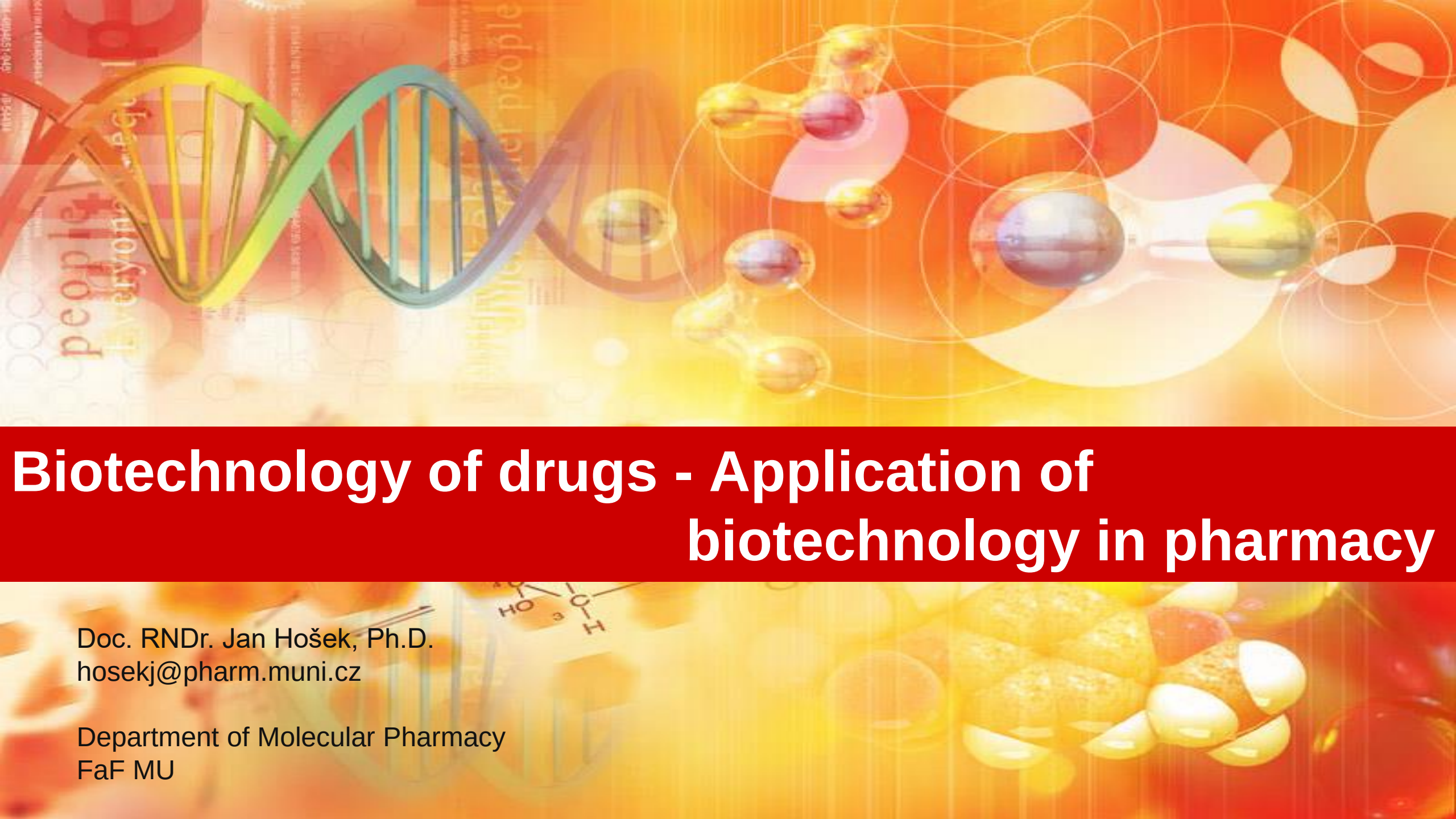

Biotechnology of drugs - Application of biotechnology in pharmacy

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Department of Molecular Pharmacy
FaF MU

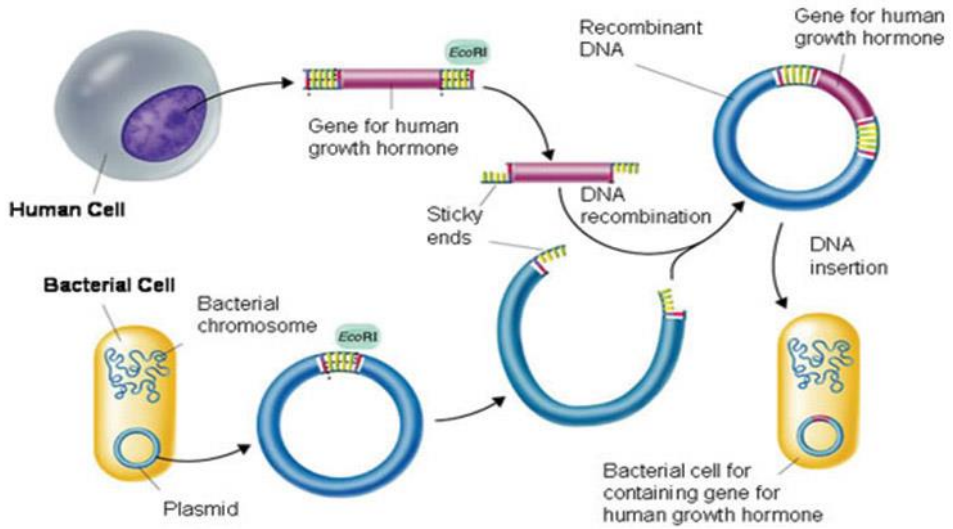
Overview of basic biotechnological productions in pharmacy

- Enzymes
- Polysaccharides
- Steroids
- Antibiotics
- Antimycotics
- Vitamins
- Alkaloids
- Hormones
- Amino acids
- Cytokinins

Two approaches

- **Products obtained by classical techniques of microbial biotechnology**

- **Products of recombinant technologies**



Products of classical biotechnology of microorganisms

The most common product = ENZYMES

Use of enzymes in pharmacy

- **preparation of medicines** – antibiotics, steroids, amino acids
- **medicines** – digestive, dissolving blood clots, etc.
- **diagnostic purposes** – part of detection kits

Output forms of the enzyme

- **Enzyme preparation**
- **Enzyme in pure form**
- **Immobilized enzyme**
- **Immobilized cells**

Examples of enzymes I.

Proteases

- trypsin, chymotrypsin, pepsin, chymosin
- papain, ficin, bromelain
- bacterial proteases (*Bacillus*)
- proteases produced by fungi (*Aspergillus*)

Glukosidases

- α-amylase, β-amylase
- produced by bacteria (*Bacillus*) and fungi (*Aspergillus*)

Use of amylases

Medicine

- facilitating the digestion of starch in dyspepsia
- reduction of meteorism before surgery and in the postoperative period

Food business

- production of beer, alcoholic beverages, spirits
- starch processing into glucose and maltose syrups and crystalline glucose
- high fructose syrups

Lipases

They catalyze the hydrolysis of triacylglycerols

Origin

- **pancreas**
- **wheat germ**
- ***Aspergillus niger*, *Rhizopus sp.*, yeast**

Application

- **part of the digestive system**
- **food industry - cheese production**

Penicilinacylase

Hydrolases cleaving bonds other than C-N

Mechanisms

- hydrolysis of penicillin to 6-aminopenicillanic acid

Origin

- *Escherichia coli, Neurospora crassa, Torula sp., Rhodotorulla sp.*

Application

- production of semi-synthetic penicillins

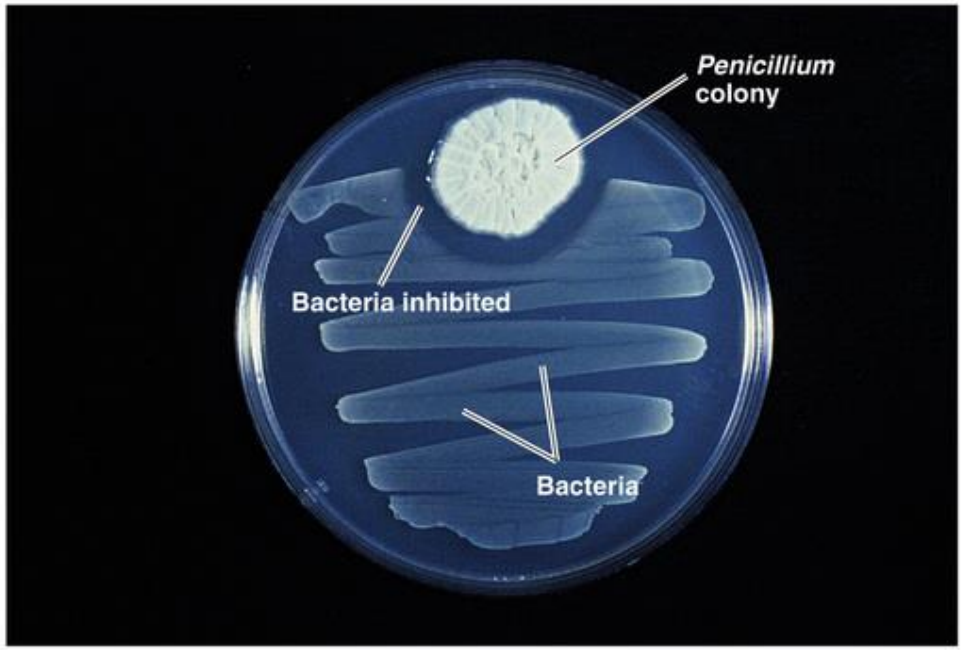
Examples of enzymes II.

Microorganisms as Biocatalysts and Enzyme Sources
DOI: http://dx.doi.org/10.5772/intechopen.90338

Microbial enzymes	Microorganism	Application
α-Amylase	*Bacillus amyloliquefaciens* *B. stearothermophilus* *B. licheniformis*	Baking, brewing, starch liquefaction Clarification of fruit juice Textile industry Paper industry
Glucoamylase	*Aspergillus niger* *A. awamori* *Rhizopus oryzae*	Beer production High glucose and high fructose syrups
Proteases	*A. usami*	
Lactase (β-galactosidase)	*Kluyveromyces lactis* *K. fragilis*	Lactose intolerance reduction in people Prebiotic food ingredients
Lipase	*Candida antarctica* *C. cylindraceae Ay30* *Helvina lanuginosa* *Pseudomonas* sp. *Geotrichum candidum*	Cheese flavor development Textile indutry Medicinal applications Use in cosmetics Use as biosensors Use in biodegradation
Phospholipases	*Fusarium oxysporum*	Cheese flavor development
Esterases	*Bacillus licheniformis*	Enhancement of flavor and fragrance in fruit juice
Xylanases	*Streptomyces* sp. *Bacillus* sp. *Pseudomonas* sp.	Clarification of fruit juice Beer quality improvement
Glucose oxidase	*A. niger* *Penicillium glaucum* *P. adametzzi*	Food shelf life important Food flavor improvement
Laccase	*Funalia trogii* *Bacillus licheniformis* *Bacillus vallismortis*	Polyphenol removal from wine baking
Pectinases	*A. niger* *A. wentii* *Rhizopus* sp.	Clarification of fruit juice
Catalase	*A. niger* *Metarhizium anisopliae* *Psychrobacter piscatorri*	Food preservation Removal of H_2O_2 from milk prior to cheese production
Peroxidase	*Streptomyces viridosporus*	Development of flavor, color and nutritional quality of food

ANTIBIOTICS

- Cell products capable of inhibiting the growth of other cells at low concentrations
- The most common producers of G+ bacteria of the genus ***Streptomyces***
- Preparation of new ATB by modification of known ATB, the so-called **mutational synthesis**



Mutation of microorganism producing ATB + suitable precursor

↓

new ATB

STEROIDES

- **the physiological effect depends on the exact position of the substituents in the basic skeleton**
- **chemical synthesis is very demanding**

Biotransformation of steroids

1) Cultivation of the microorganism - increase in biomass
2) Addition of steroid, subsequent biotransformation
3) Isolation into an organic solvent
4) Purification by chromatography and crystallization

Microorganisms as Biocatalysts and Enzyme Sources
DOI: http://dx.doi.org/10.5772/intechopen.90338

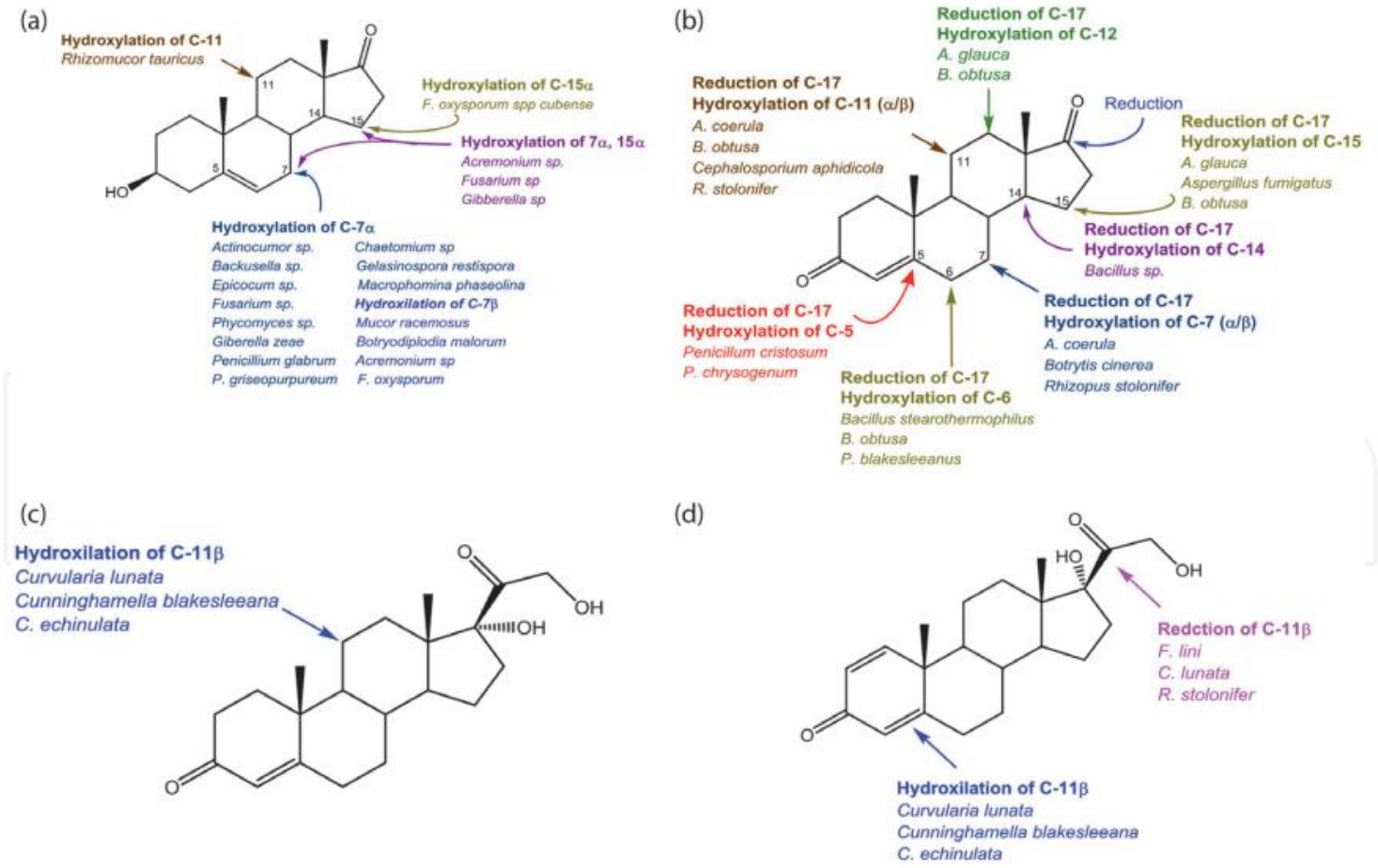


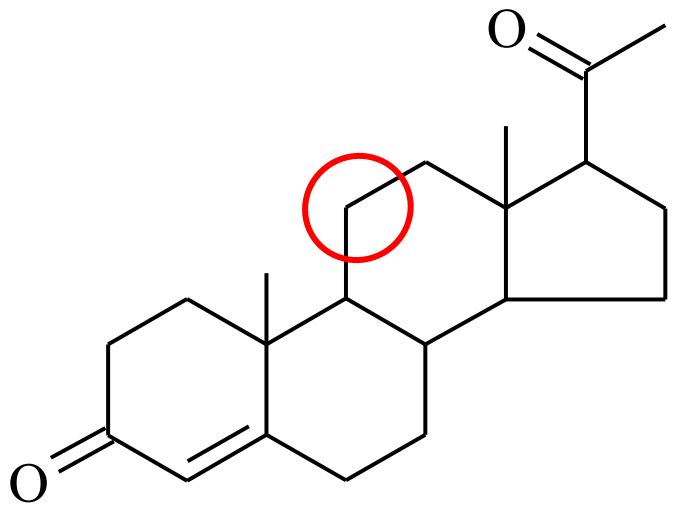
Figure 11.
*The ability of different fungi to transform DHEA (**125**), testosterone, cortexolone (**126**) and prednisone (**127**). (a) Hydroxilation of 3β-hydroxy-5-androsten-17-one (DHEA) by various microorganisms. (b) Reduction of C-17 and hydroxilation of testosterone by various microorganisms. (c) Hydroxylation of cortexolone (**123**) by various microorganisms. (d) Reduction and hydroxylation of prednisone (**126**) microorganisms.*

Examples of biotransformations I.

11α-hydroxylation

Preparation of 11-α-progesterone

Rhizopus nigricans, R. arrhizus, Aspergillus ochraceus



11β-hydroxylation

Preparation of cortisol

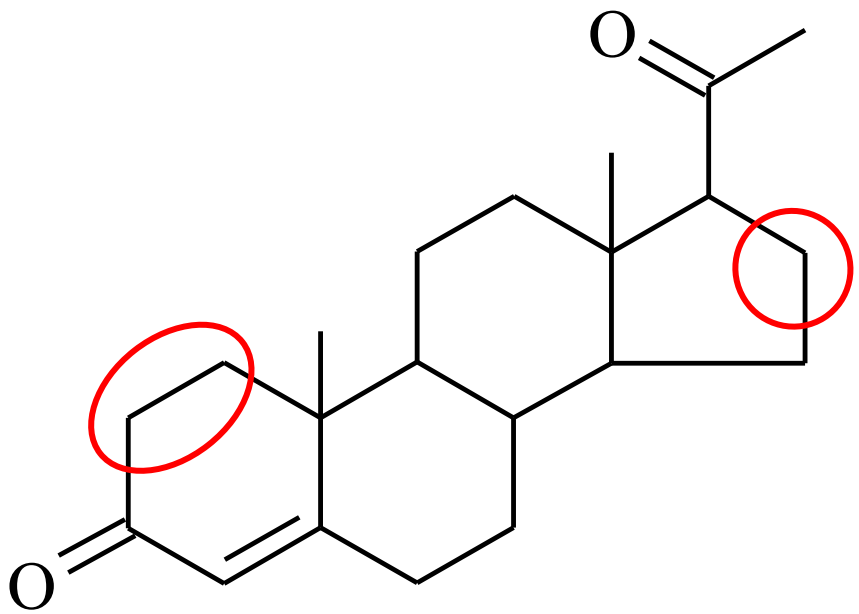
Curvularia lunata, Cunninghamella blakesleeana

Examples of biotransformations II.

16α-hydroxylation

Preparation of 16α-hydroxy-9α-fluoroprednisolone (triamcinolone)

Streptomyces roseochromogenes



dehydrogenation between C1-C2

Bacillus lensus, Arthrobacter simplex

Preparation of prednisone, prednisolone, triamcinolone, 6-methylprednisolone, dexamethasone…

ERGOT ALKALOIDS

Sources

***Claviceps purpurea* (growing on rye)**

***Claviceps paspali* (submerged cultivation)**

Sclerotia of *Claviceps purpurea* on rye

Spores on sclerotium

VITAMINS

essential animal nutritional factors

Preparation

- chemical synthesis
- isolation from natural material
- microbial biosynthesis
- biotransformation

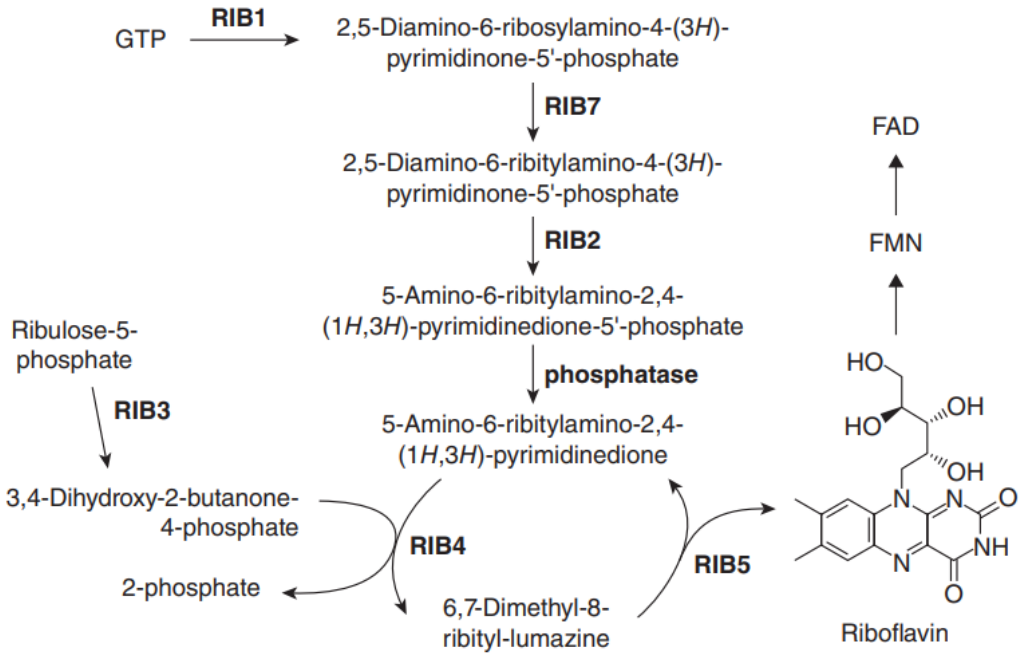


Fig. 21.6 Biosynthetic pathway of riboflavin in *Ashbya gossypii*. GTP = guanosine 5'-triphosphate; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; RIB (1–5 and 7) = riboflavin biosynthesis gene(s).

Biotechnologically produced vitamins

- riboflavin (B_2)
- cobalamin (B_{12})
- ascorbic acid (C)
- ergosterole (D_2, D_3)

- provitamin A
- provitamin D

DOI: 10.1533/9780857093547.2.571

AMINOACIDS

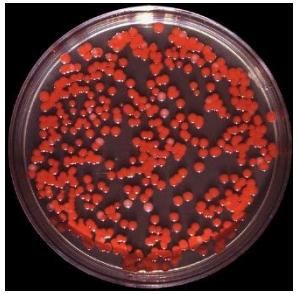
- Chemical synthesis
- Isolation from natural sources
- Enzymatic transformations
  - cultivation of MO containing the relevant enzyme
  - cell separation
  - a substrate intended for enzymatic conversion is added to the cells
- Biosynthetically - cultivation of microorganism - isolation of aminoacids from culture

Naturally occurring bacteria

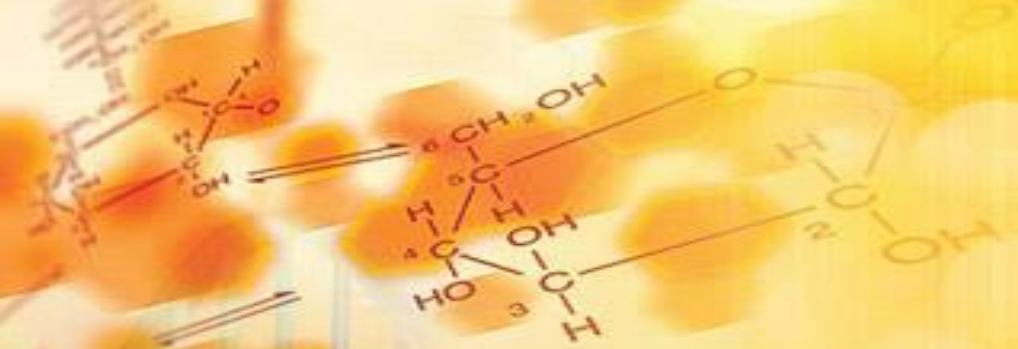
- ***Corynebacterium***
- ***Brevibacterium***
- ***Micrococcus***

Recombinant strains

- ***Escherichia coli***
- ***Serratia marcescens***



Recombinant drugs



Types of recombinant drugs

- the leader is the USA (Food and Drug Administration)
- dozens of drugs, all protein-based

  - hormones
  - enzymes
  - hematopoietic growth and coagulation factors
  - cytokines and interferons
  - antibodies and their derivatives
  - vaccines
  - other products

Recombinant hormones

amino acid sequence alteration

changes in pharmacokinetics

different biological effects

broader treatment approaches

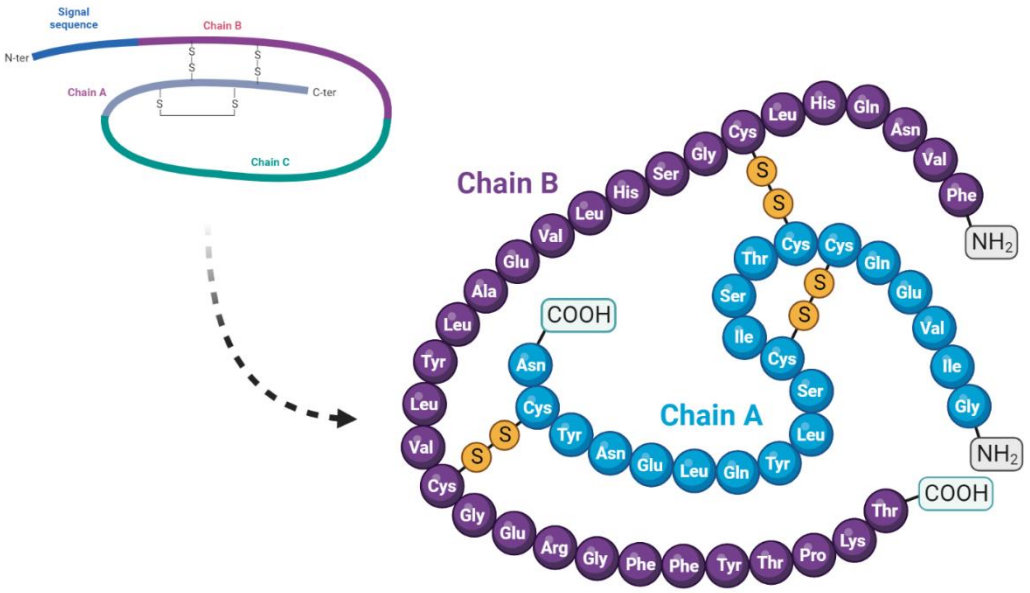
Recombinant hormones were the first

y. 1979 - Goeddel et al. - production of insulin and somatotropin by *Escherichia coli*

y. 1982 (28.10.) - insulin - the first clinically used recombinant hormone (USA)

(Humulin-R, Eli Lilly and Genentech)

y. 1985 - somatotropin



https://myendoconsult.com/learn/insulin-physiology-and-clinical-applications/

Insulin analogs

Lispro (HUMALOG)

- reversed order of lysine and proline at positions B28 and B29
- production in *Escherichia coli*
- short-acting insulin

Aspart (NOVORAPID)

- substitution of proline with aspartic acid in position B28
- *Saccharomyces cerevisiae*
- short-acting insulin

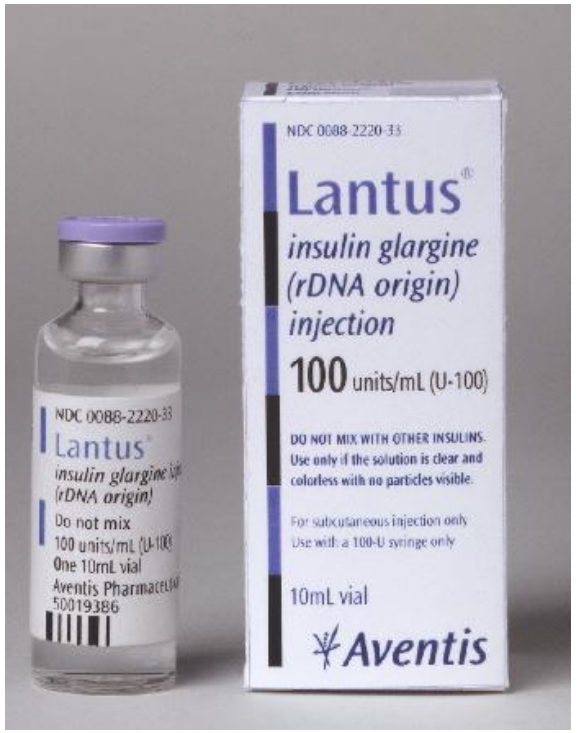
Other types of insulin

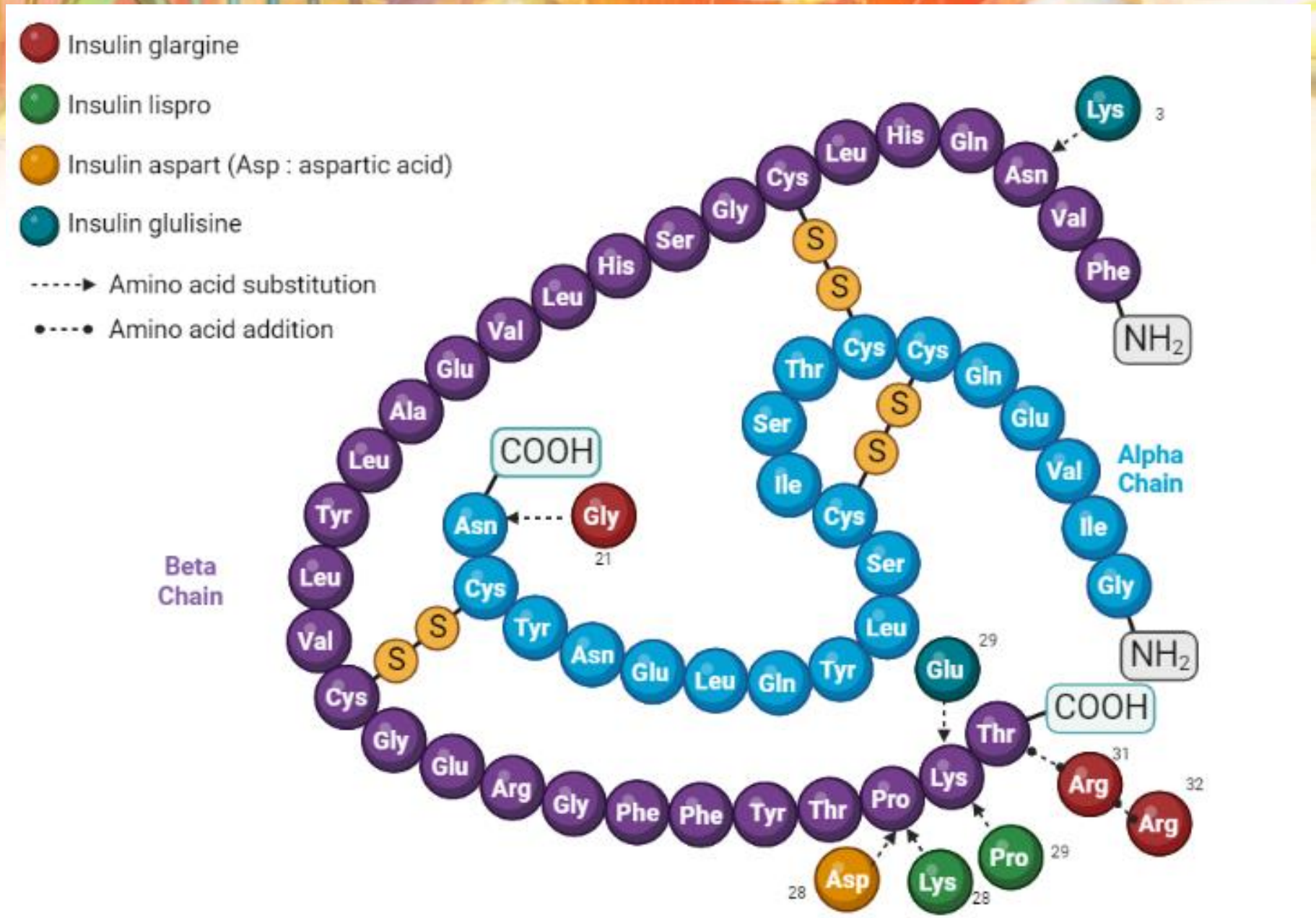
Glargin (LANTUS)

- addition of 2 arginines to the C-terminus of chain B and substitution of glycine for asparagine at A21
- *Escherichia coli*
- prolonged effect

Detemir

- removal of threonine at B30 and acylation (myristic acid) of lysine at position B29
- prolonged effect

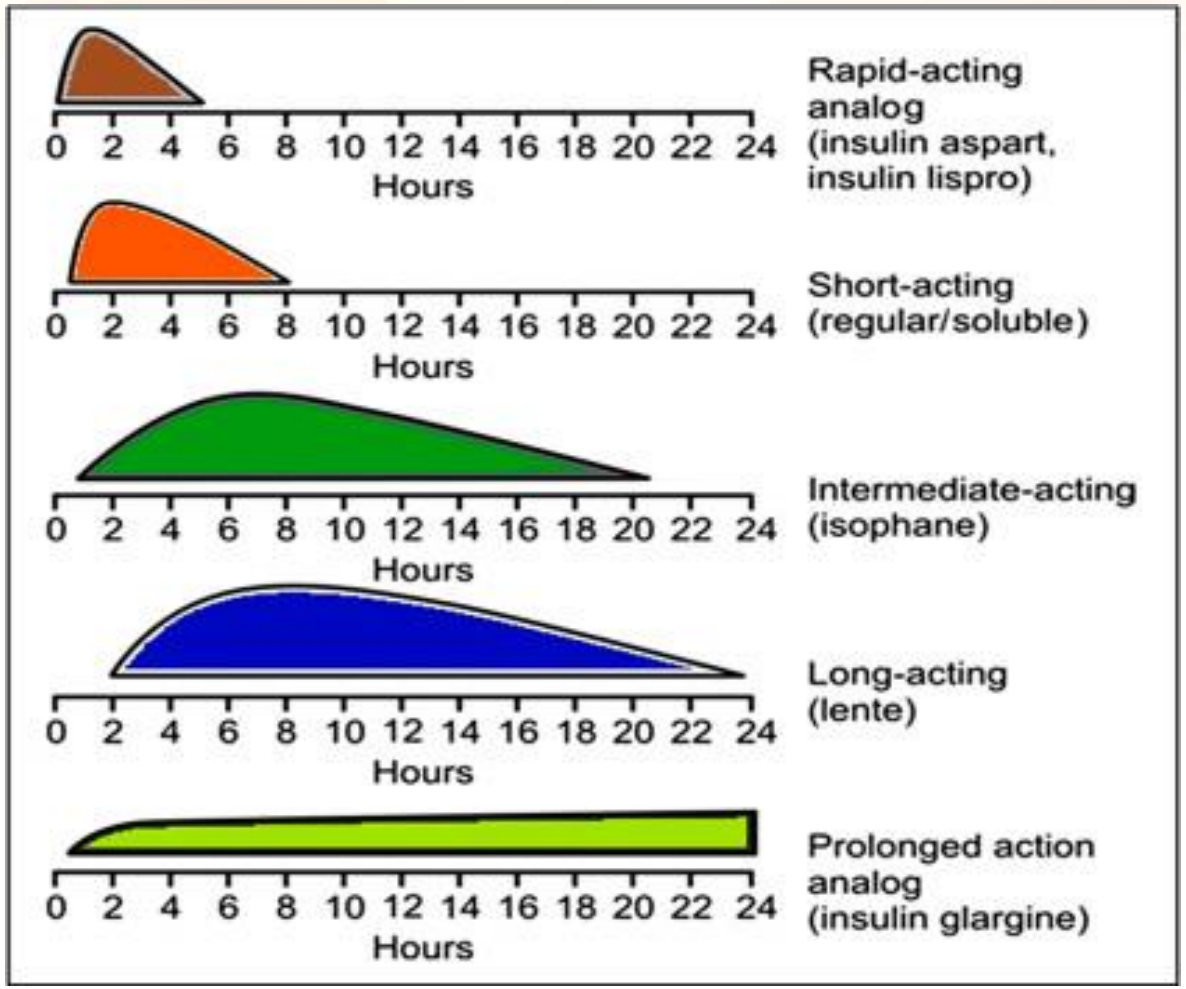




https://myendoconsult.com/learn/insulin-physiology-and-clinical-applications/

Advantages over human short-acting insulins

- faster and more regular absorption from the subcutaneous tissue
- they best mimic prandial secretion
- treatment of patients from 3 years of age
- shorter biological effect - lower risk of hypoglycemia



Glucagon

A polypeptide hormone (29 AA)

Effects

- **glycogenolytic**
- **hyperglycemic**
- **relaxation of GIT smooth muscle**

Preparation

- ***Escherichia coli***
- ***Saccharomyces cerevisiae***

Indication

- **hypoglycemia**
- **radiological examination – inhibition of GIT movement**

Somatotropin

A species-specific polypeptide (191 AA)

Effects

- growth stimulation
- increases proteosynthesis
- reduces proteocatabolism

Indication

- growth disorders

Preparation

- *Escherichia coli* (since the late 1980s)

Folitropin

follicle stimulating hormone (FSH)
Two subunits – alfa (92 AA), beta (111 AA)

Production

- **CHO cells**

Indication

- **anovulatory cycles**
- **amenorrhea**
- **disorders of spermatogenesis**

Recombinant enzymes

Preparation of medicines

- antibiotics, steroides, aminoacids

Drugs

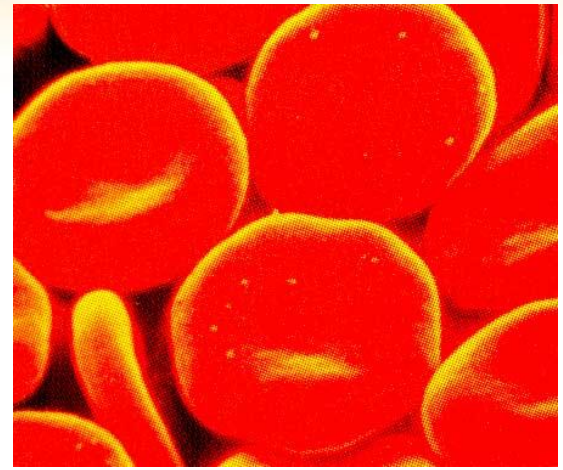
- digestive (dissolving blood clots, treating leukemia)

Diagnostic and R&D purposes

- Taq polymerase
- Restriction endonucleases

Hematopoietic and growth factors

Epoetin α (165 AA, glycosylated)



Effects

stimulation of blood cell formation

Preparation

mammalian cells

Indication

treatment of anemia

Cytokines and interferons

Interleukin IL-2

Effects

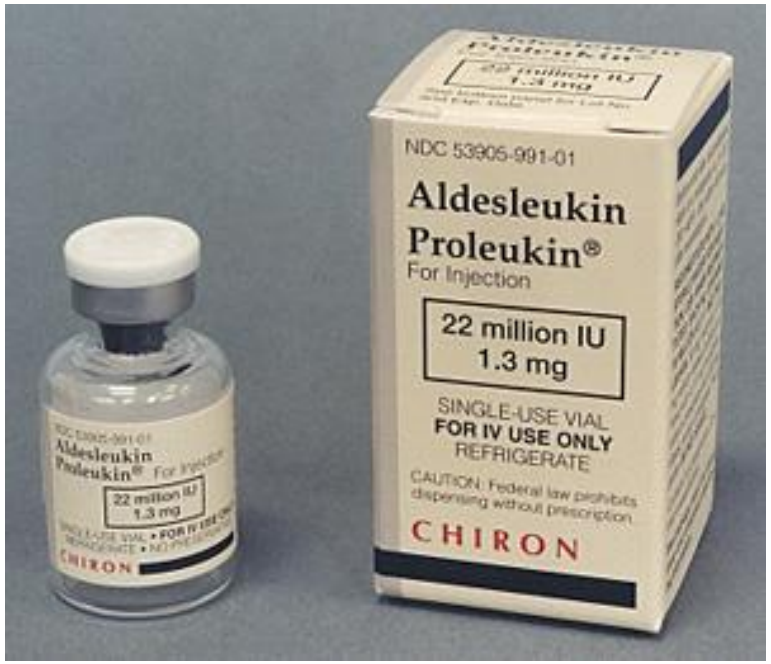
immunomodulating effects

Preparation

Escherichia coli

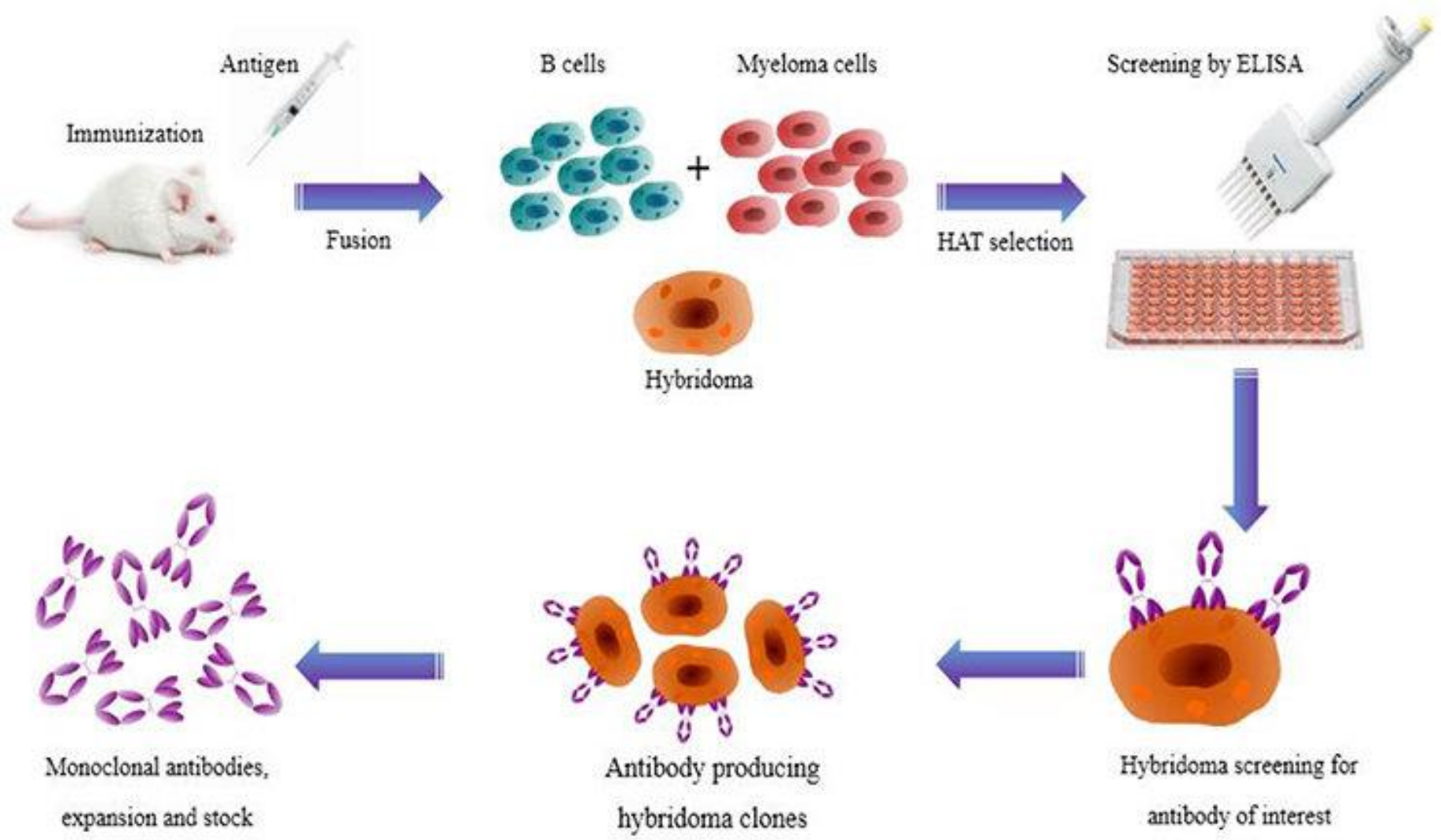
Indication

cancer therapy



Interferons α, β, γ

Antibodies

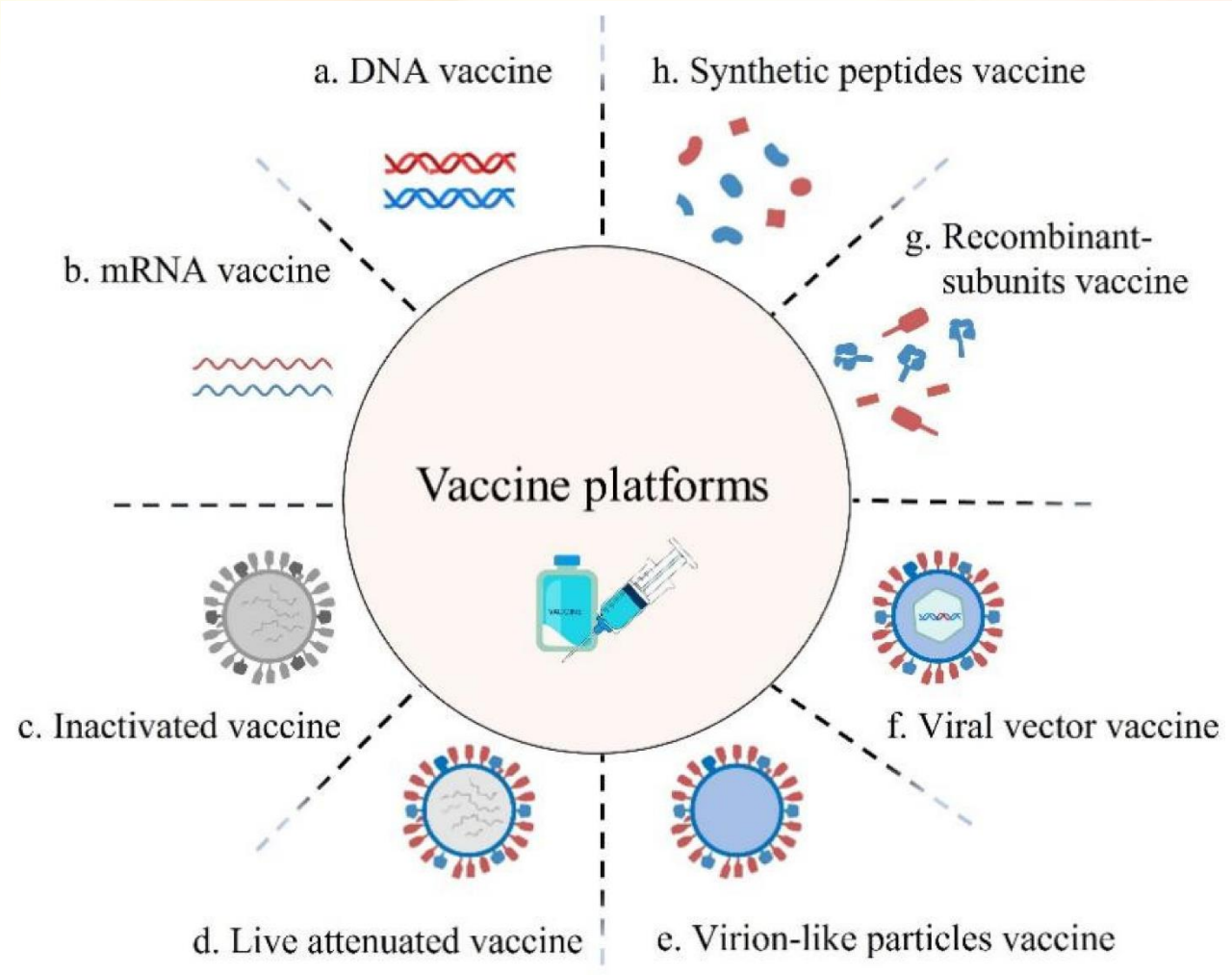


DOI:10.3389/fmicb.2017.00495

Recombinant vaccines

There are several production strategies

- **Methods of reverse genetics**
- **Recombinant subunit vaccines**
- **Production of "virus-like" particles**
- **DNA and RNA vaccines**
- **Vaccines based on viral vectors**



http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3548171/

Transgenic animals

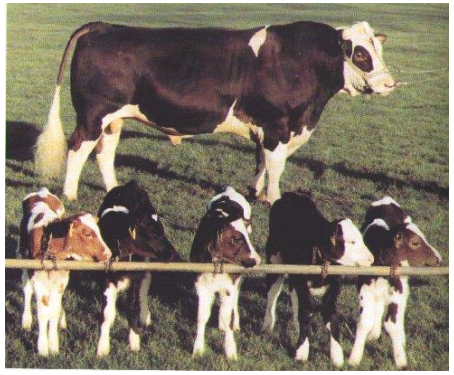
sheep Polly

- blood clotting factor IX
- treatment of hemophilia

goats

- antithrombin III
- prevents the formation of blood clots
- GTC Biotherapeutics, USA

cows

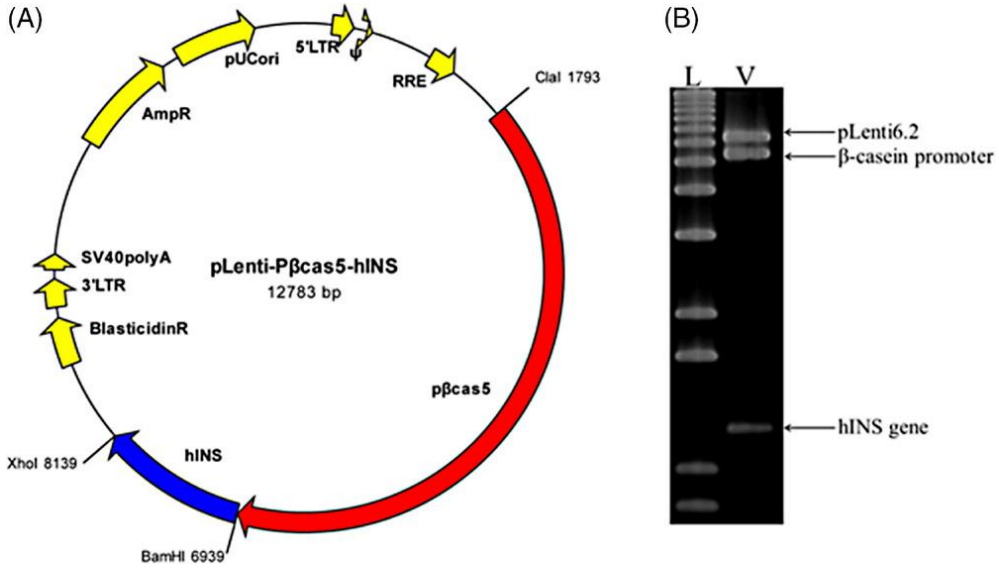


- human lactoferrin
- Pharming, Netherlands

News of 2024

- Production of human pro/insulin in cow milk

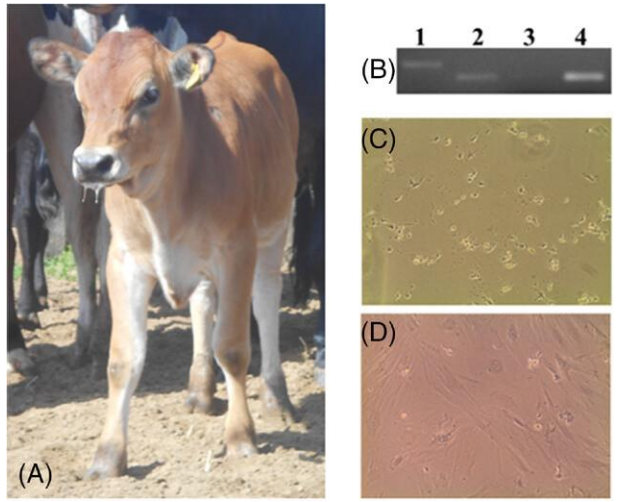
Biotechnology Journal, Volume: 19, Issue: 3, First published: 12 March 2024, DOI: (10.1002/biot.202300307)



Schematic of the lentiviral vector constructed for mammary gland-specific human insulin expression and restriction map analysis. (A) Schematic of the lentiviral vector constructed. (B) The restriction map for the vector, L- ladder 1 kb plus (Life Technologies < Waltham, MA); V- constructed vector digested with BamHI, ClaI, and XhoI restriction enzymes. The fragment of the hINS gene was generated by cleavage of the BamHI and XhoI enzymes, while BamHI and ClaI generated the fragment of the β-casein promoter, and the ClaI and XhoI enzymes generated the fragment from the original vector pLenti6.2-GW/EmGFP (7.833 kb). The size of the β-casein promoter used was 5.335 kb and the human proinsulin gene fragment was 1.193 kb.

TABLE 1. In vitro development and pregnancy rates.

Oocytes, *N*	1st PB extrusion, *N* (%)	Reconstructed, *N*	Fusion, *N* (%)	Blastocysts, *N* (%)	Transfers, *N*	Pregnancy (60 days), *N* (%)
74	53 (71.6)	53	42 (79.2)	11 (26.2)	10	1 (10.0)



Transgene analysis. (A) Photo of the transgenic calf. (B) PCR analysis of the transgene: 1- DNA ladder, 2- transgenic calf, 3- non-transgenic cow, 4- lentiviral vector constructed. (C) Nonmodified bovine fibroblasts at 5 days incubated with 8 μg mL^{-1} blasticidin. (D) Fibroblasts from the transgenic calf at 8 days incubated with 8 μg mL^{-1} blasticidin.

Isolation and purification of recombinant products

Biotechnology products for therapeutic use must be precisely specified, especially when intended for parenteral administration.

Protein Production

Protein Expression
- Strain selection
- Codon optimization
- Fusion systems
- Co-expression
- Mutagenesis
- Isotope labeling

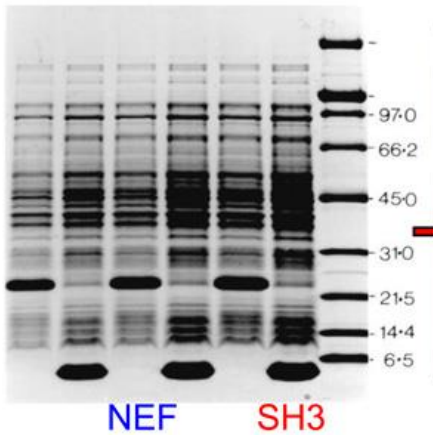
Protein Purification
- Soluble Protein
- Folding of insoluble protein
- Membrane proteins
- Cleavage of fusion moieties

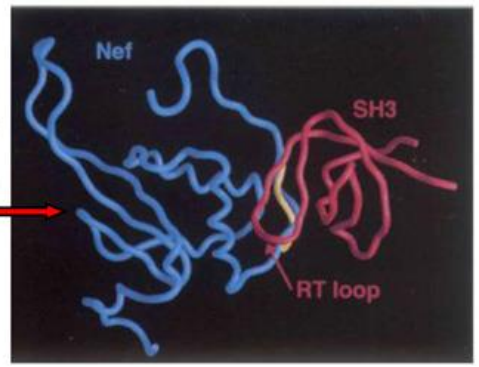
Characterization

Physiochemical
- Mass Spectrometry
- Physical homogeneity
- Association stoichiometry
- Conformational stability

Functional
- Biochemical
- Immunochemical
- Protein interactions

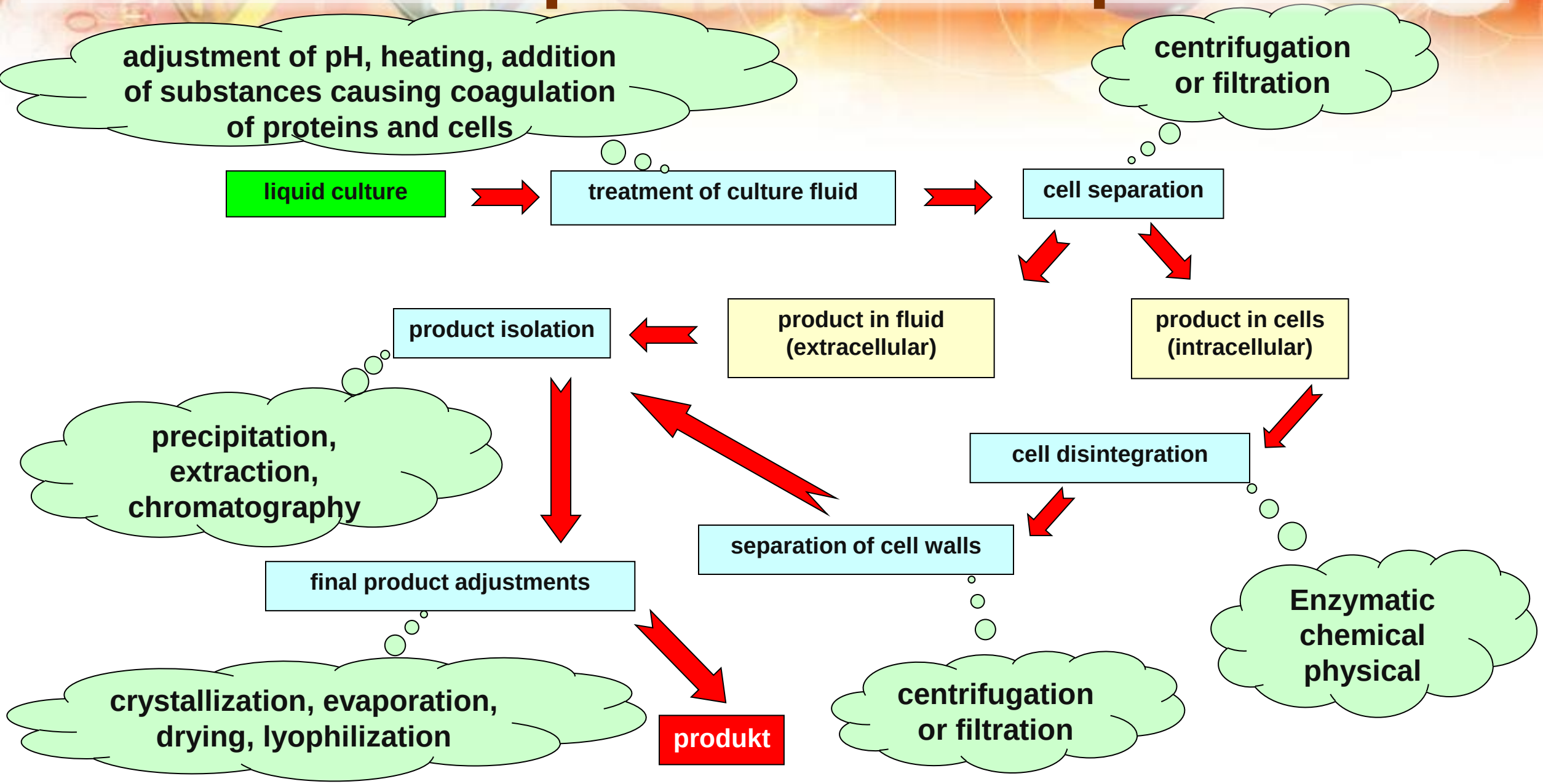
Structure
- Protein crystallization
- X ray analysis
- NMR
- Electron Microscopy



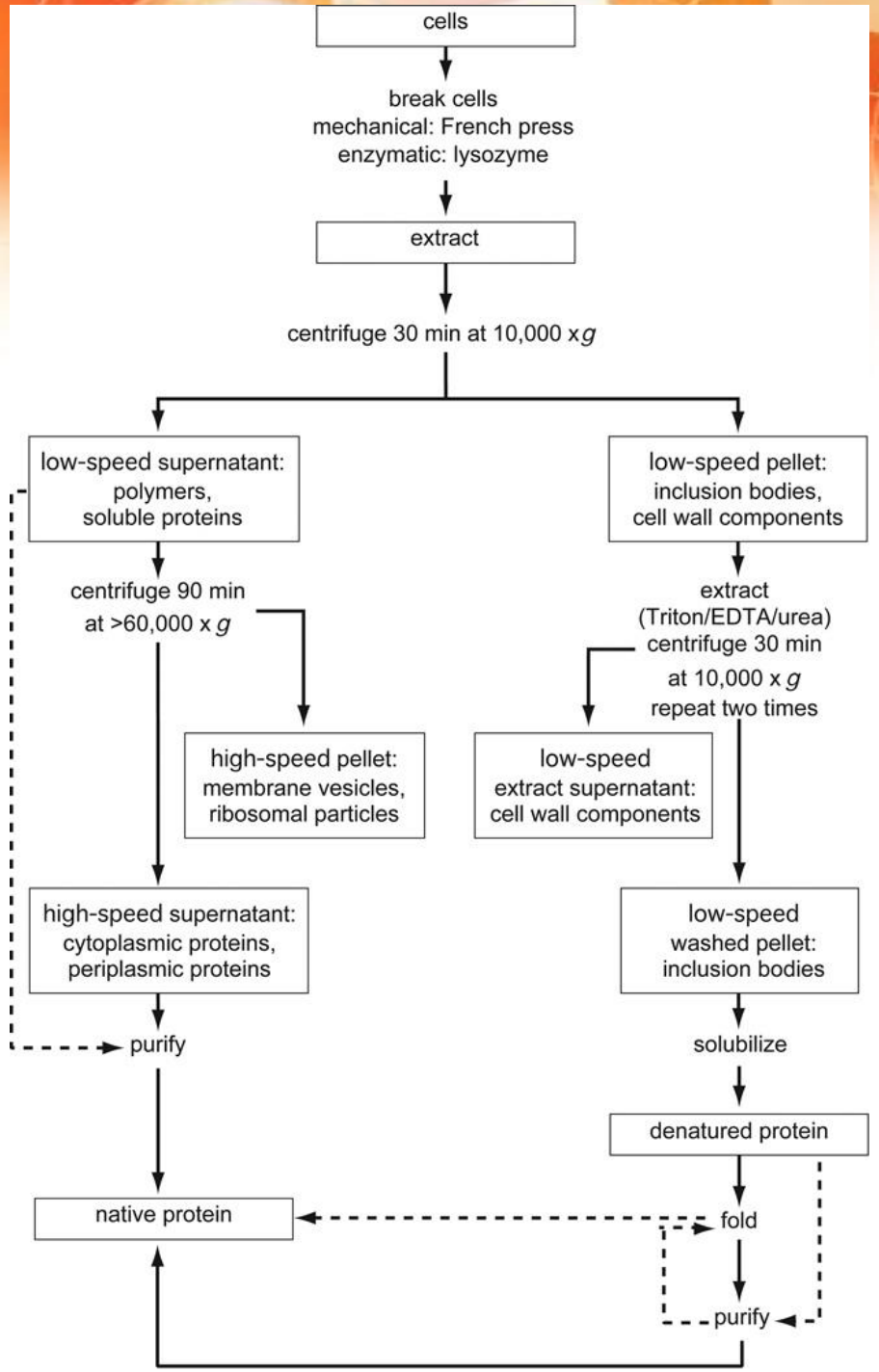


doi: 10.1002/0471140864.ps0601s80

Isolation and purification of products

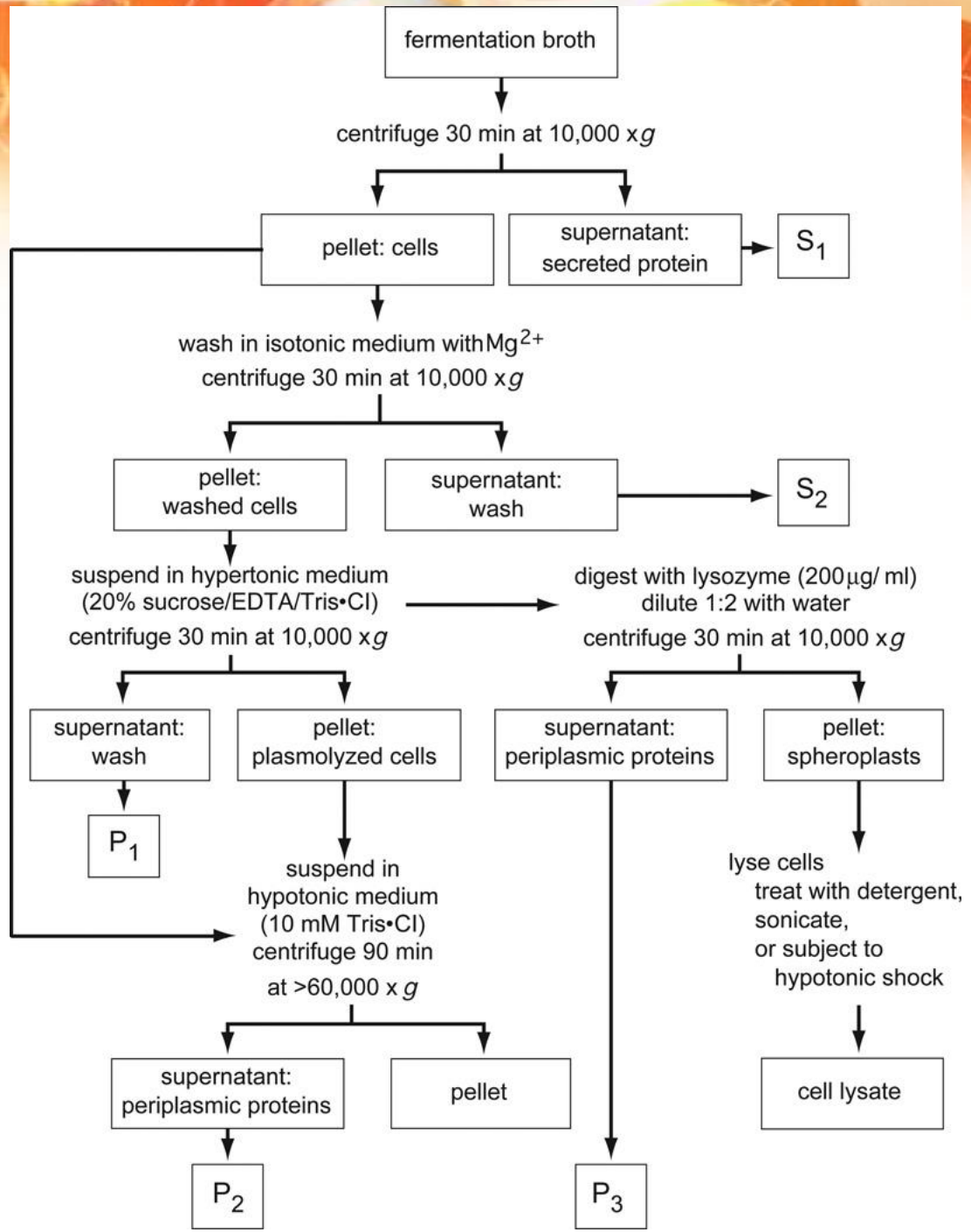


Differential centrifugation of *E. coli* cell lysates. Cells are broken with a French press or by lysozyme treatment. Insoluble (inclusion body) proteins, from either the cytoplasm or periplasm, are located in the low-speed pellet, which is subjected to preextraction to remove outer membrane and peptidoglycan material. Inclusion bodies are extracted from washed pellets with strong protein denaturants such as guanidine·HCl. The solubilized protein, which is denatured and reduced (free sulfhydryl residues), is either directly folded and oxidized (disulfide bonds formed) or purified before folding. Soluble proteins (from the periplasm and cytoplasm) are located in the low-speed and high-speed supernatants. The latter can be used directly for chromatography, whereas the former requires clarification by other techniques such as ammonium sulfate fractionation or membrane filtration.

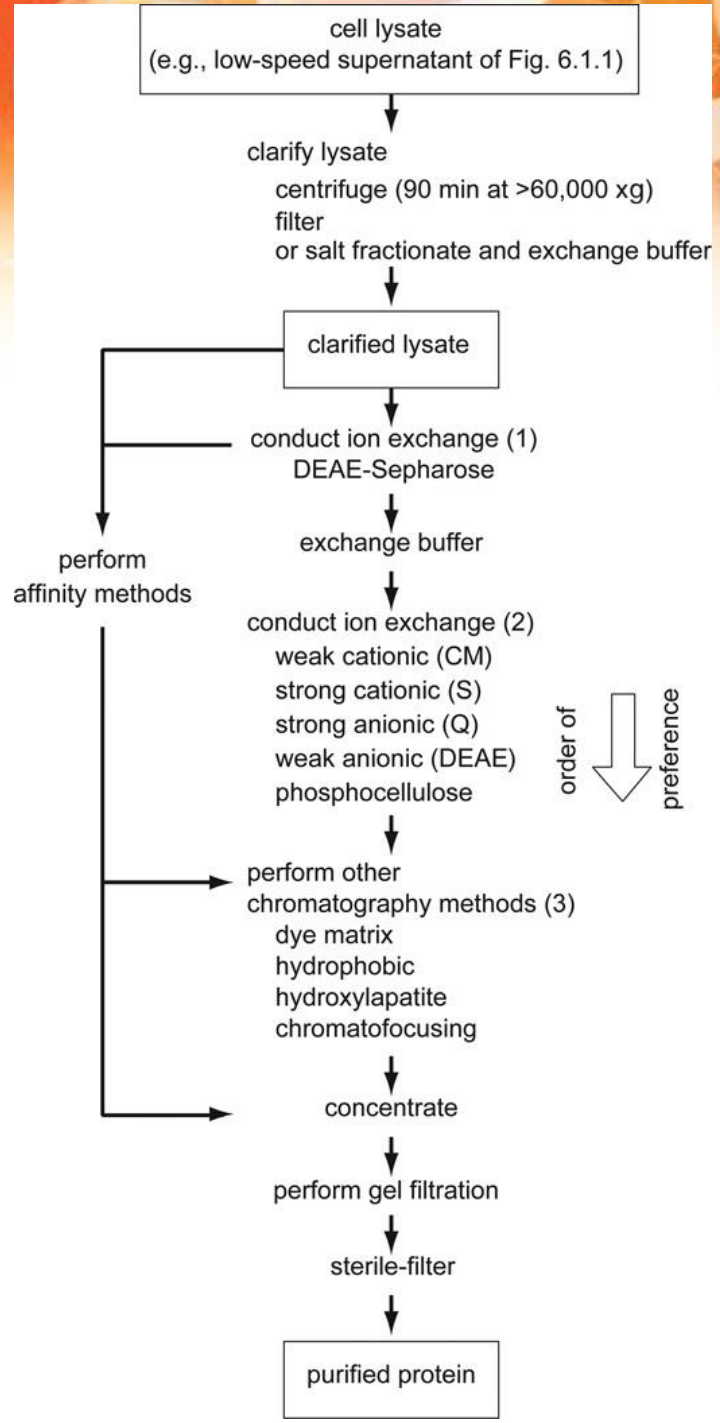


Curr Protoc Protein Sci. 2015; 80: 6.1.1–6.1.35.
Published online 2015 Apr 1. doi: 10.1002/0471140864.ps0601s80

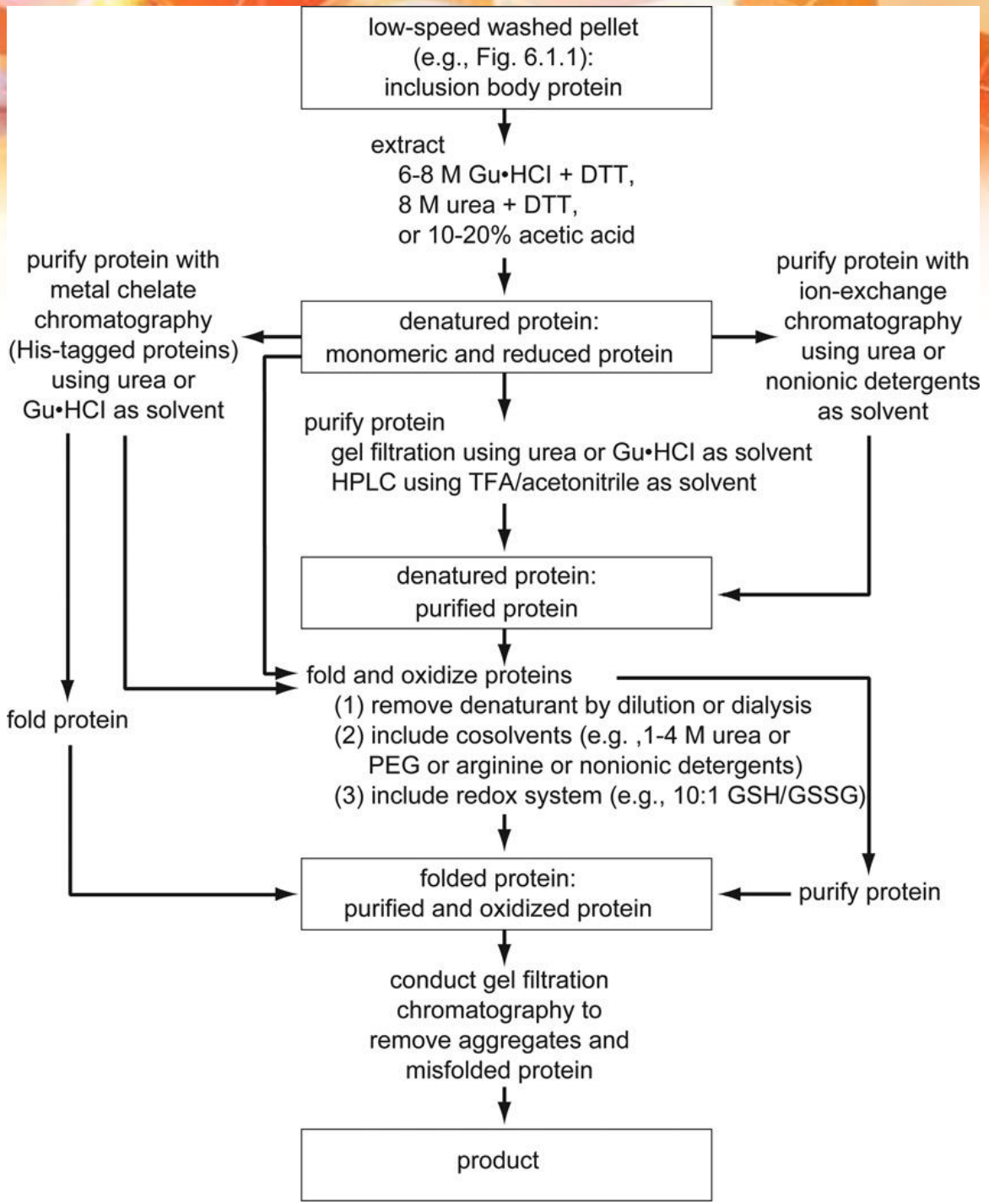
Localization of secreted and periplasmic proteins in *E. coli*. Periplasmic protein produced via a secretion vector can leak into the medium and be recovered by centrifugation (supernatant, S1) or filtration. Washing cells with an isotonic solution such as lightly buffered 0.15 M NaCl or 0.25 M sucrose can also release protein (S2). The compartmentalized periplasmic proteins are released by isotonic shock treatment by directly suspending normal cell paste or plasmolyzed cell paste into hypotonic medium. Plasmolyzed cell paste is derived by suspending cells in hypertonic medium and then pelleting. (In hypertonic medium the cell contracts, separating the inner membrane from the cell wall, and is said to be osmotically sensitized.) The hypertonic wash often releases protein (P1). The supernatant from shocked cells (P2) will contain constitutive *E. coli* proteins and the recombinant product. Osmotically sensitized cells can also be treated with lysozyme to fragment the outer membrane, thus releasing periplasmic proteins (P3).The pellet from the lysozyme treatment contains spheroplasts (cells with fragmented outer membranes), which are easily disrupted by detergents, sonication, or hypotonic shock to release cytoplasmic proteins.

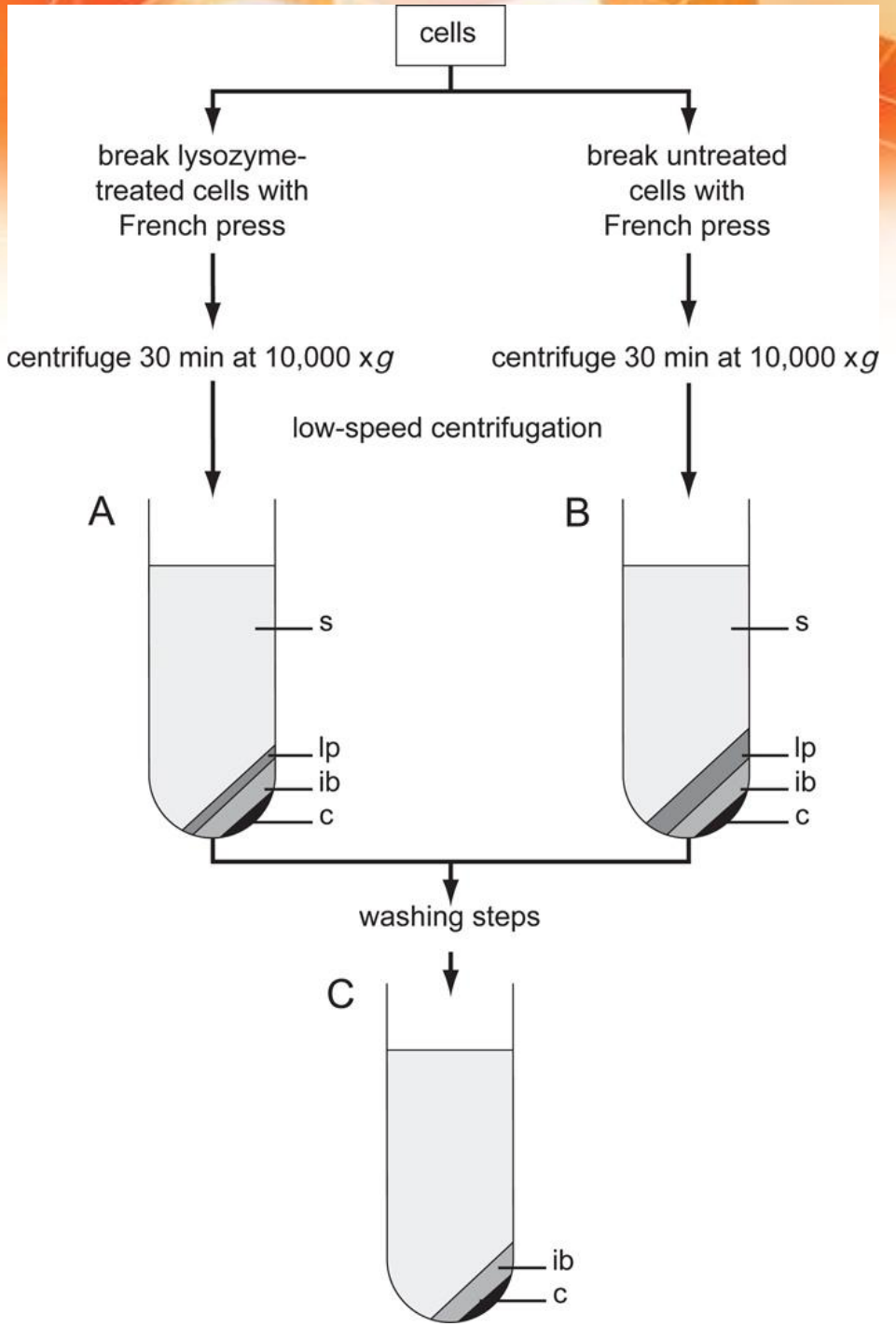


Purification of soluble proteins from bacterial cell and other cell lysates. Abbreviations for ion-exchange resins are as follows: CM, carboxymethyl; DEAE, diethylaminoethyl; Q, quaternary ammonium; S, methyl sulfonate. The order of preference for the stages of ion-exchange (2) and other methods (3) is based on the author's opinion and does not necessarily represent a consensus view. On the other hand, the use of a DEAE-based matrix at an early stage (1) is common practice. Affinity methods (see text and Chapter 9) can be performed at any stage following clarification of the lysate.



Folding and purification of inclusion body proteins from *E. coli*. The protein is extracted with protein denaturants such as guanidine·HCl (Gu·HCl), urea, or an organic acid. The reductant dithiothreitol (DTT) is included to prevent artificial disulfide bond formation (especially intermolecular bonds). The denatured protein can be purified by various methods and then folded, or it can be directly folded. Typically, some purification (e.g., gel filtration in Gu·HCl) prior to folding is recommended as it often results in higher folding yields. Protein folding and oxidation are carried out concurrently. Disulfide bond formation is catalyzed by low-molecular-weight thiol/disulfide pairs such as reduced (GSH) and oxidized (GSSG) glutathione. GSH/GSSG ratios of 5:1 to 10:1 are normally used, which are similar to those found in vivo in the endoplasmic reticulum (Hwang et al., 1992). A cosolvent is included to maintain solubility during folding. Folded protein is purified if necessary (purification is usually needed if the protein is directly folded). Gel filtration is a useful final step for removing aggregated and or misfolded protein.





Preparation of washed pellets using lysozyme and the French press. Cells are broken with the French press with or without prior treatment with lysozyme. After low–speed centrifugation using a fixed-angle rotor, the contents of the centrifuge tubes have the characteristics shown. The contents of tubes A and B are labeled: s, supernatant; lp, loose pellet; ib, inclusion body protein; and c, unbroken cells and large cellular debris. The loose pellet material is derived from the outer cell wall and outer membrane (see text for further details). After washing the insoluble material (UNIT 6.3), the pellet should consist mainly of the inclusion body layer (tube C), and the supernatant should be fairly clear.

Contaminating components

- Quality is usually expressed in terms of purity and reproducibility.
- The purity of pharmaceutical preparations is usually higher than 99% if they are administered parenterally.
- This also applies to protein therapeutics, for which the resulting purity is highly dependent on the purification process.

Origin of contaminating components of protein pharmaceuticals

- Host organism
- Product
- Process

Contaminating components of the host organism

- Viruses
- Host proteins and DNA
- Glycosylation variants
- Variant N and C termini
- Endotoxins from Gram negative bacteria

Contaminating components originating from the product

- Amino acid substitutions and deletions
- Protein denaturation
- Conformational isomers
- Dimers and aggregates
- Variants of disulfide bridges
- Deaminated proteins
- Protein fragments

Contaminating components created within the process

- Growth medium components
- Purification reagents
- Metals
- Materials of purification columns

How to get rid of virus particles?

Category	Method	Example
Virus inactivation	Heat	Pasteurization
	Radiation	UV irradiation
	Dehydration	Lyophilization
	Crosslinking substances, denaturation, decomposition	β-propionolacton, formaldehyd, NaOH, org. solvents, detergents
	Neutralisation	Specific antibodies
Virus removal	Chromatography	Ion exchange, immunoaffinity
	Filtration	nanofiltration
	Precipitation	cryoprecipitation

What is nanofiltration?

Filtration through 15 nm membranes, which can capture even the smallest known non-enveloped viruses such as bovine parvovirus

Bacteria as a contaminating component

- **Bacteria from cell cultures can be easily removed by filtration**
- **Work sterile, clean areas (air sterilization)**
- **Work under antibiotics, which are then difficult to remove !!**

What else does bacteria do?

Pyrogens – substances of different sizes with different structures

- **Sensitive people - fever with fatal consequences**
- **Remove by ion exchange chromatography**
- **Hot air sterilization of instruments**

Mycoplasmas

- **It changes the characteristics of metabolism, growth, cell lifespan, etc.**
- **Remove with gentamicin or ciprofloxacin**

Cellular DNA as a contaminating component

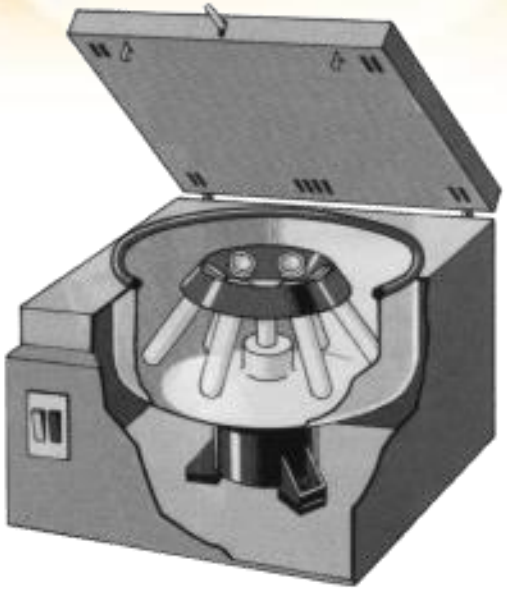
- When mammalian cells are used, DNA fragments are present in the medium
- What is a safe level?
- The European Pharmacopoeia recommends that the amount of DNA in the final preparation of the therapeutic protein does not exceed 100 pg to 10 ng daily dose depending on the type of culture system

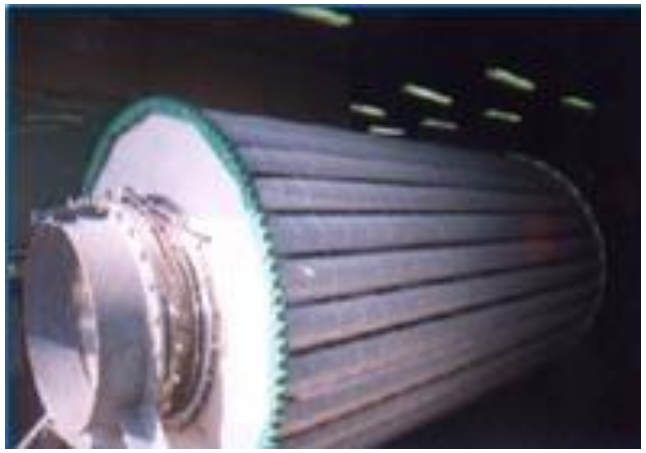
Contaminating proteins

- "Foreign" proteins can be recognized as antigens → an immune response that the recombinant protein is not responsible for
- Sourced by host cell or medium
- Beware of recombinant protein variants!
- Detection of contaminating proteins immunologically

Cell separation

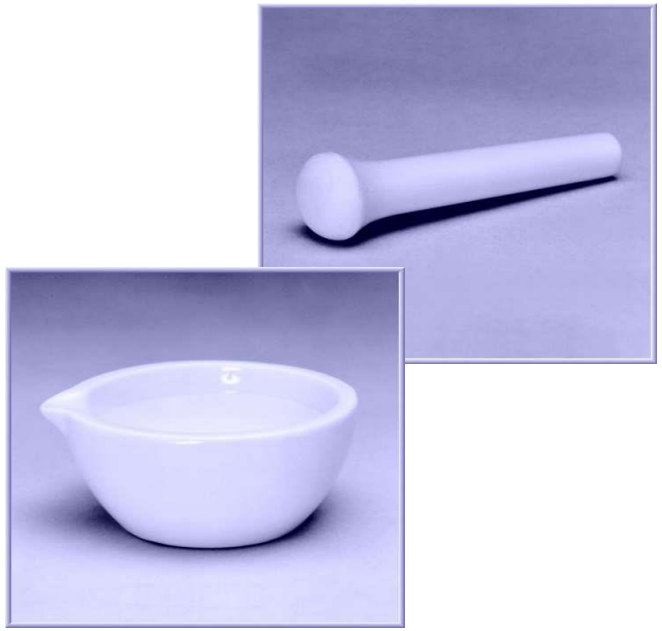
- centrifugation
- filtration (drum rotary filters)





Cell disintegration

- **enzymatic: lysozyme**
- **chemical: alkali, detergents**
- **physical: osmotic shock, cell crushing with abrasives, ultrasound**



Separation of cell walls

- centrifugation
- filtration

Isolation of the product from the liquid

- **Extraction**
  - **system of two immiscible solvents**
  - **in protein isolation PEG and dextran or PEG and specific salts such as K_3PO_4 or NH_4SO_4**
- **Precipitation**
  - **salting out proteins NH_4SO_4**
  - **precipitation with organic solvents (EtOH, acetone, ...)**
- **Chromatographic methods (gel, ionex, bioaffinity, adsorption)**
- **Electromigration methods (electrophoresis, isoelectric focusing, isotachophoresis)**

Final adjustments of the product

Evaporation

- vacuum evaporators
- beware of thermolabile substances
- for thermolabile enzymes, plate (film) evaporators are most suitable

Drying

- removal of water and volatile substances from the product
- belt, hazel, drum, spray dryers
- **fluid-current dryers** (blowing the material with warm air) - common **in the pharmaceutical industry**

Protein separation methods

Isoelectric focusing

- Movement of proteins in a pH gradient after application of an electric field
- Proteins migrate to the so-called "isoelectric point"

Two-dimensional electrophoresis

- Separation by isoelectric focusing - based on electric charge
- Electrophoresis separation - based on size

Chromatography

- Separation based on the different permeability of proteins filling chromatographic columns

Protein identification methods

Mass spectrometry

- Based on the ratio of mass to charge of ionized molecules (MALDI-TOF)

Antibody detection

- Polyclonal antibodies
- Monoclonal antibodies

- Western blot
- Immunoprecipitation, immunocytochemistry and immunohistochemistry

Chromatographic methods

- It is a collective designation for a group of physical-chemical separation methods
- It is used for the separation and analysis of complex mixtures of substances
- In all types of chromatographic separation, the molecules of the analyzed substance are divided between the so-called stationary and mobile phase
- The separation is based on the different distribution of the components of the mixture between the mobile and stationary phases

Types of chromatographic methods I.

According to the purpose of use

- analytical chromatography
- preparative chromatography

According to the physico-chemical principle

- adsorption chromatography
- partition chromatography
- ion exchange chromatography
- gel chromatography
- (bio)affinity chromatography

Types of chromatographic methods II.

According to the state of the mobile phase

- liquid chromatography
- gas chromatography

According to the arrangement of the stationary phase

- column chromatography
- capillary chromatography
- thin layer chromatography (thin layer chromatography)
- paper chromatography

Chromatography

- **biologically active substances form an extensive group of compounds with special functions**
- **changes in pH, ionic strength, concentration of metal ions, cofactors, etc. can result in a large effect on the isolated biologically active molecules**
- **in order not to lose their biological activity during isolation, it is necessary to use the mildest possible separation methods**

Purification strategy – I.

- low concentration of biologically active substances
- a mixture of many similar substances

First stage of isolation = adsorption

- biospecific affinity chromatography
- at physiological pH values, most proteins are negatively charged → sorption to annex

Another level of isolation

- gel chromatography
- electrophoretic methods

Purification strategy – II.

Isolation of a pure biologically active substance is most often achieved by a combination of several separation methods

When choosing a purification scheme, care should be taken not to repeat methods based on the same separation principle

Adsorption chromatography

It is based on different adsorption of substances on the surface of the sorbent, forming the stationary phase

- **Substances that are more strongly bound by sorption forces under the given conditions are adsorbed in individual sections more often and for longer than other substances**
- **Stationary phase sorbents differ in polarity or acidity**
  - **non-polar - activated carbon, polar acidic silica gel (SiO_2)**
  - **polar - basic hydrated alumina or magnesium oxide**
- **Mobile phase - solvent mixtures (... chloroform, ethanol, ...)**
  - **For gas adsorption chromatography, nitrogen or helium**

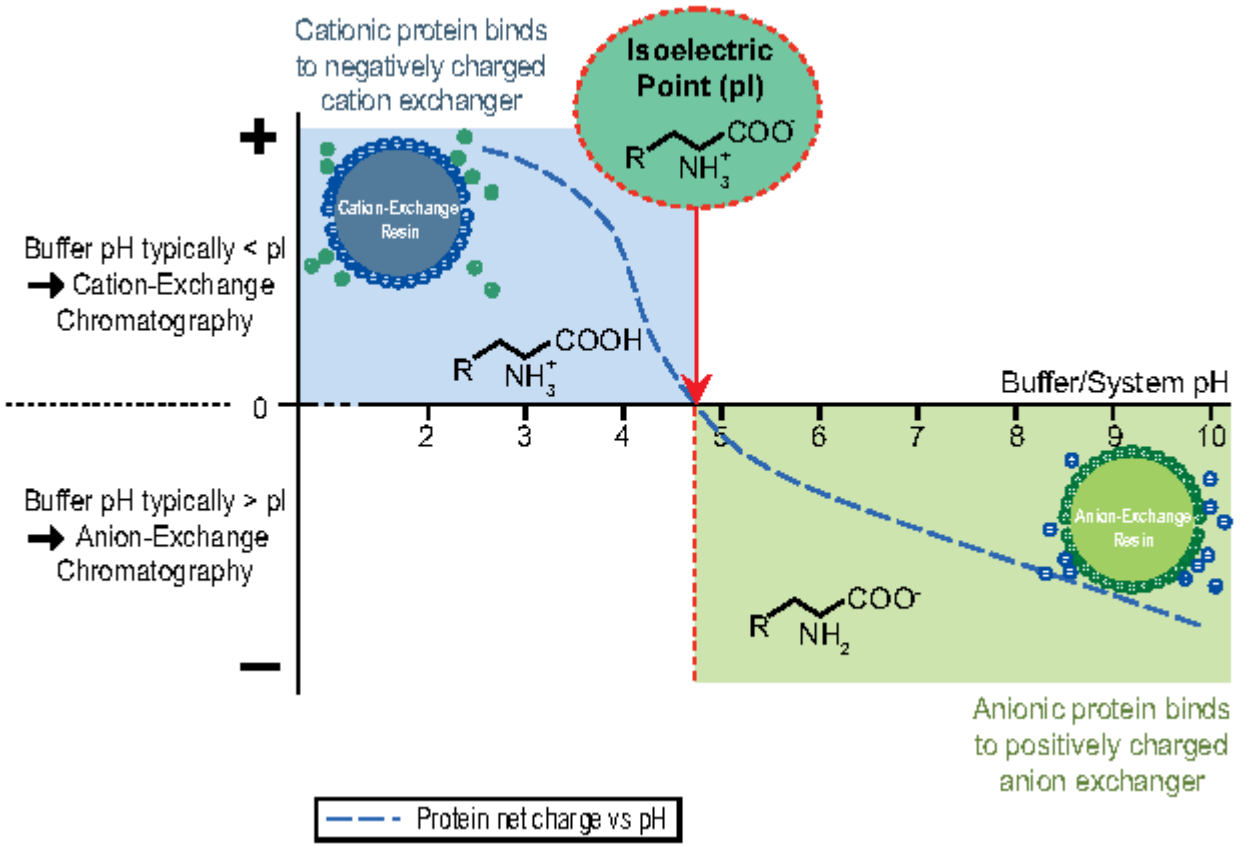
Partition chromatography

It is based on the different solubility of the separated substances in two different liquids, i.e. on different values of the partition coefficient ($\alpha = c_m/c_s$)

- **One of the liquids used is the mobile phase, the other is then anchored on some carrier and thus forms the stationary phase**
- **Higher value of α = stronger binding to the stationary component = slower flow through the column**
- **Normal phase = anchored stationary phase is water**
- **Inverted phase = anchored stationary phase consists of low polar organic liquids**
- **Carriers – SiO_2, glass, polymers, starch, cellulose, etc.**

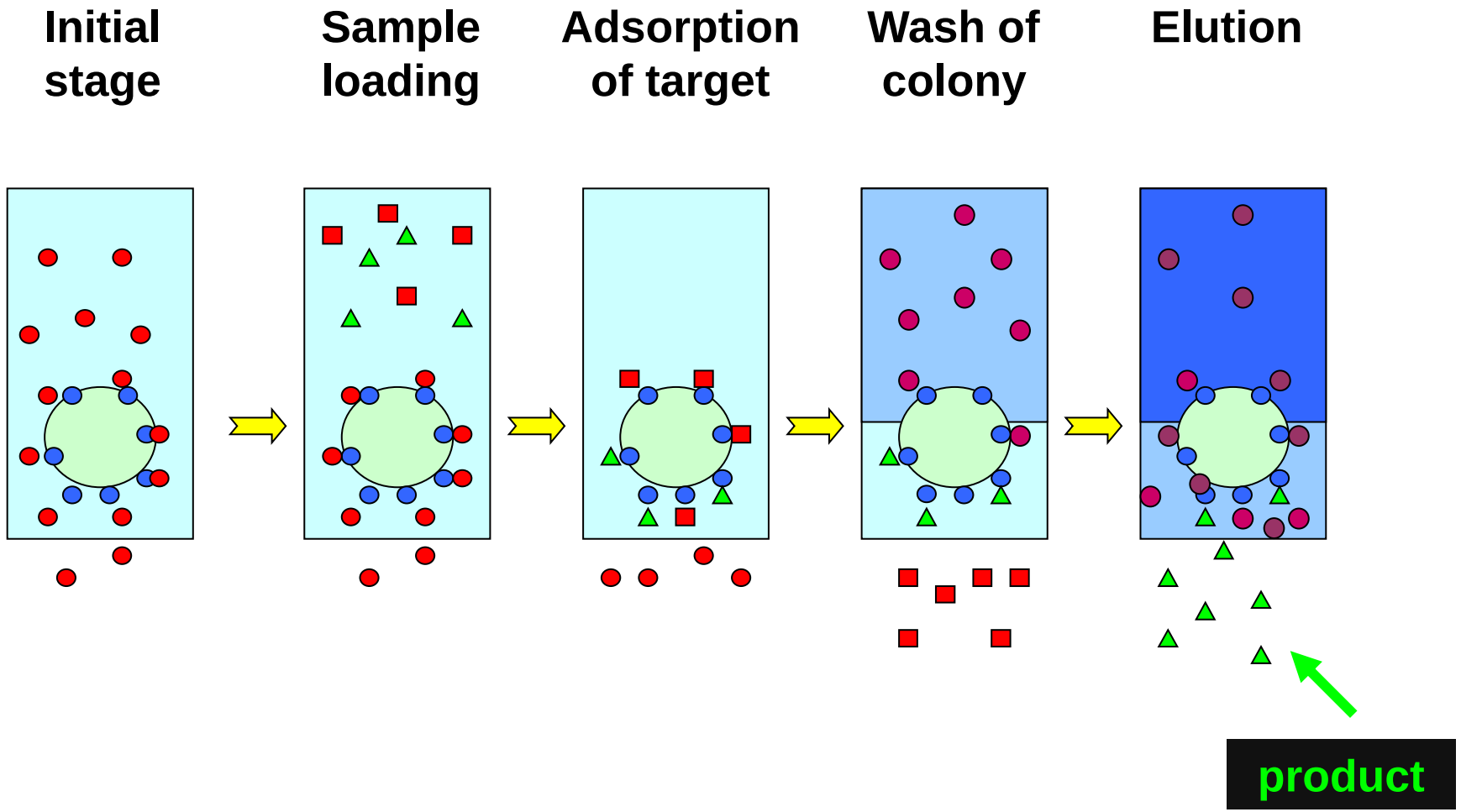
Ion exchange (ionex) chromatography

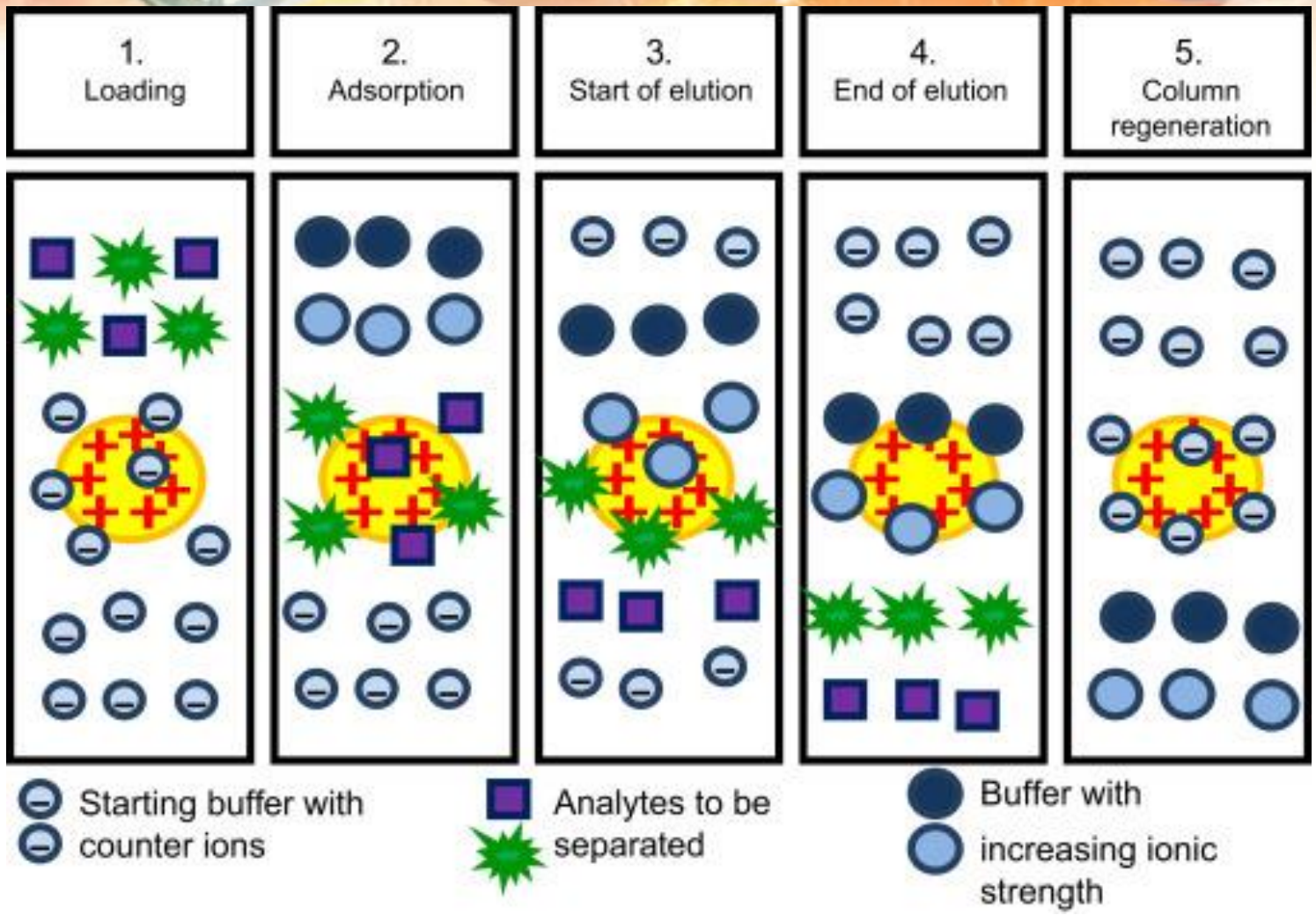
- **the basis is the reversible exchange of ions between the mobile liquid and the stationary phase**
- **stationary phase - ionexics (anex or katex)**



Gendeh, Gurmil et al. “Exploration of pH-Gradient Ion-Exchange Chromatograp y High-Resolution Protein Separations in Biotechnology d Pr teomics.” (2012).

Course of ion exchange chromatography





https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/ion-exchange-chromatography

Gel chromatography

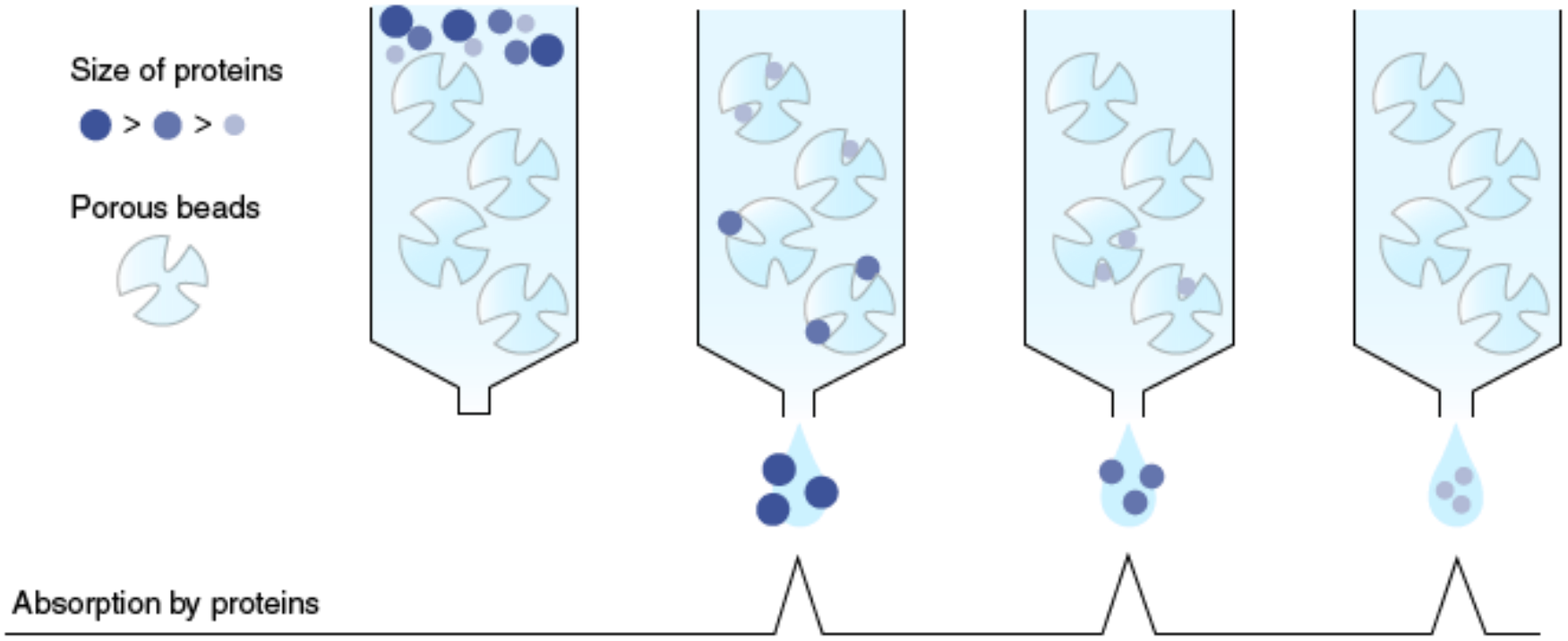
Separation of macromolecules based on the different size of individual substances on a porous stationary phase (gel filtration)

<u>Stationary phase</u> - inert porous material saturated with liquid

- **agarose**
- **cross-linked dextran (Sephadex)**
- **polyacrylamide (BioGel P)**
- **cellulose (Cellufin)**
- **materials based on silica gel or porous glass**

Gel chromatography

In other words: it is based on the different permeability of the holes and hollow niches on the particles of the stationary phase for different sized particles of the partitioned mixture



https://www.mblbio.com/bio/g/support/method/chromatography.html

Principle of gel chromatography

When a mixture of substances passes through a porous stationary phase, it happens that

- **small molecules are able to diffuse into the pores of the matrix and their movement is thus slowed down**
- **large molecules are not captured and pass through the matrix faster - the larger the molecule, the faster it passes out of the column**

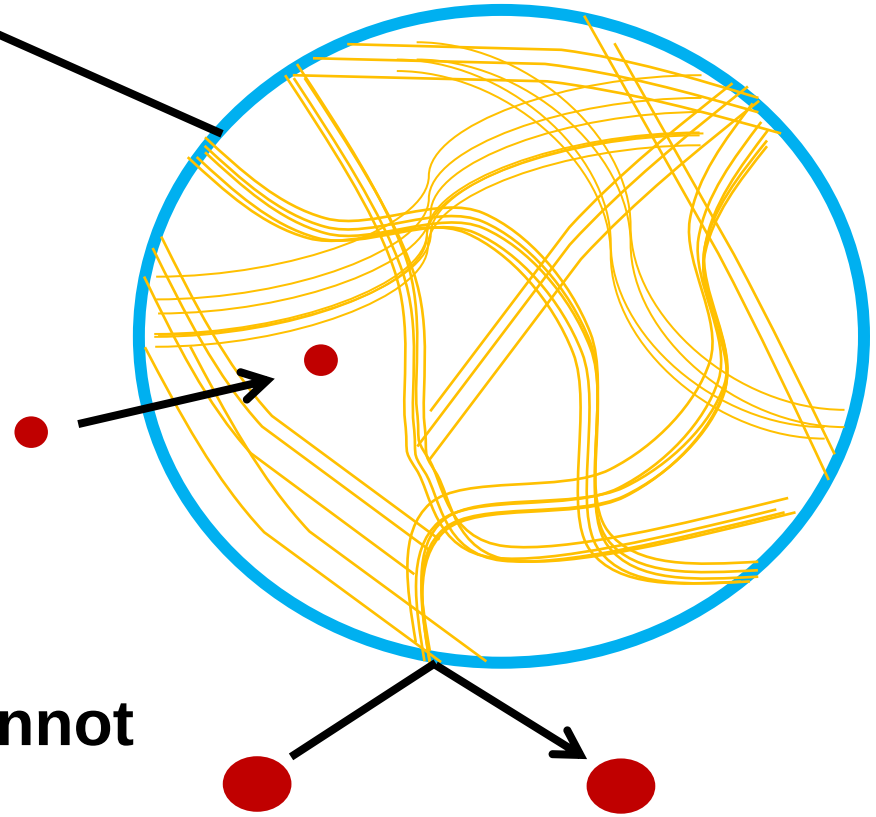
- **Small molecules are also washed out of the column by successive washing of the mobile phase**
- **It is important that there are no bonds between the split solution and the matrix or denaturation of the split material**

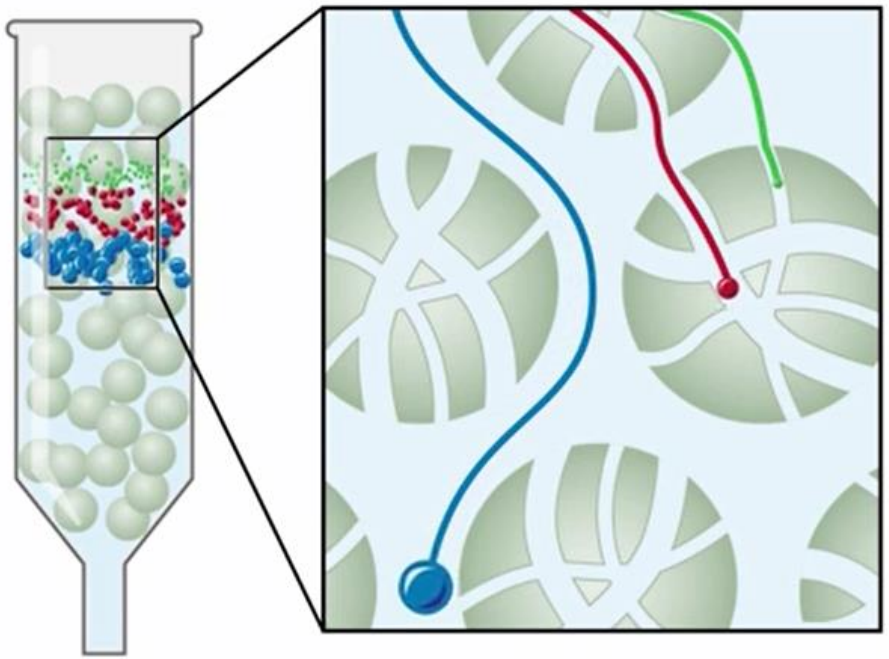
Gel filtration

Cross-linked gel particles

Small molecules can "enter" inside

Large molecules cannot "enter" inside





https://www.adareng.com/es/articulo/chromatography-types/n-41

Affinity (bioaffinity) chromatography

based on the exceptional property of biologically active substances to form strong specific reversible complexes with other complex-forming compounds, so-called affinity ligands

enzyme – substrate, cofactor – effector, antibody – antigen, hormone – receptor, etc.

The principle of the isolation method is the interaction of the isolated protein with a ligand bound to a solid support

Ligand =

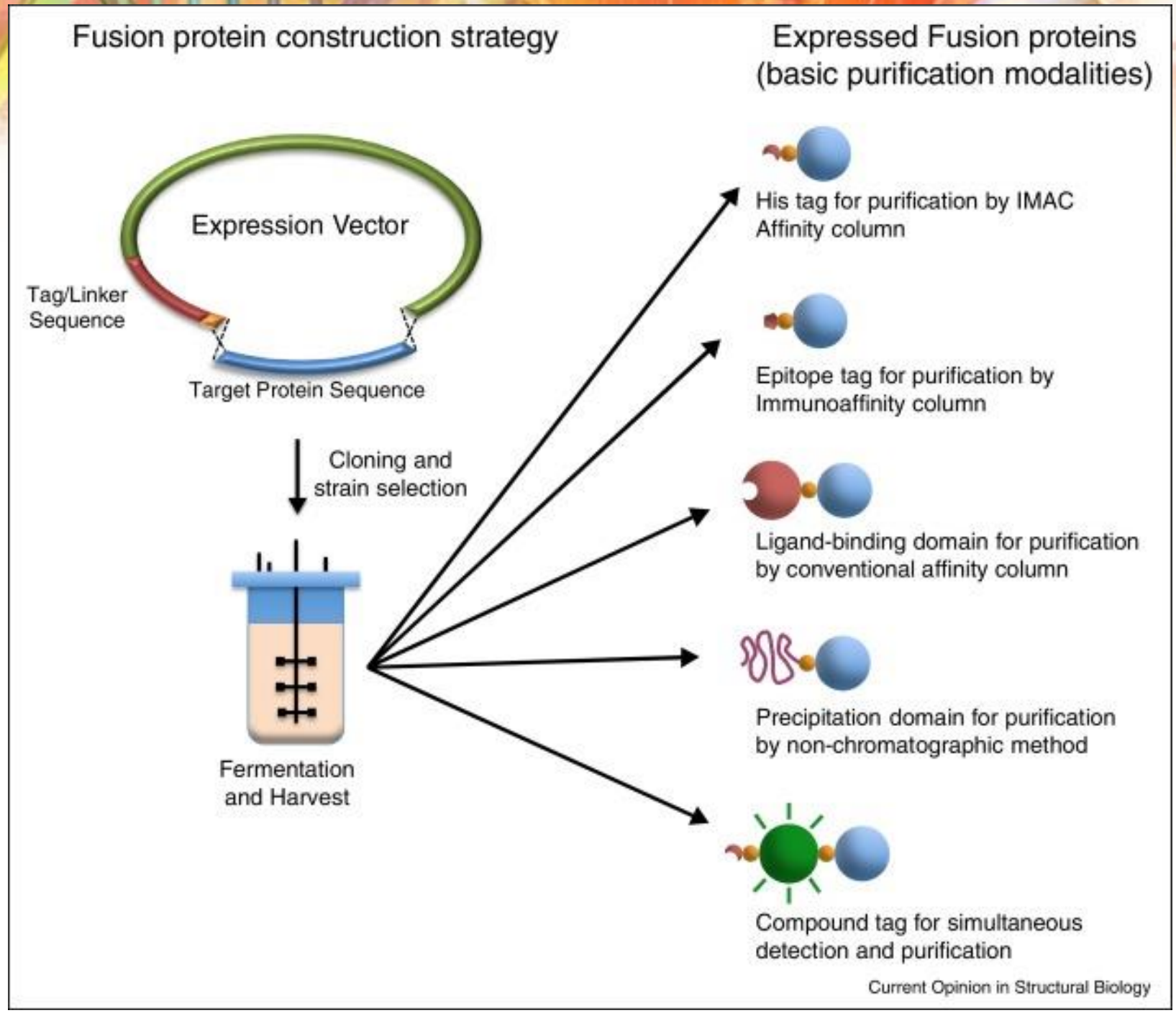
a compound that forms a biospecific reversible complex with a given isolated substance

Ligands in affinity chromatography

Any compound that forms a biospecific reversible complex with the given isolated substance can be used as a ligand

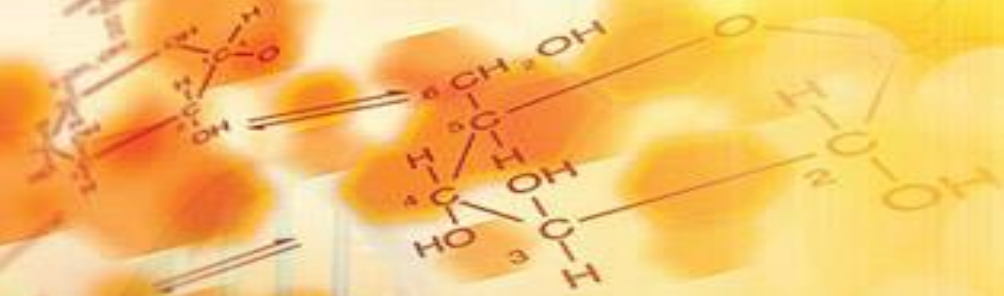
- **it must contain a functional group that is covalently bound to a solid support**
- **it must have sufficient affinity for the isolated substance**

- **immobilized pyridine or adenine nucleotides**
- **dyes with an anthraquinone structure**
- **immobilized hemoglobin or casein for proteolytic enzymes**



http://dx.doi.org/10.1016/j.sbi.2014.04.006

Protein identification methods



Mass spectrometry

Molecular weight determination method

- **It divides proteins (peptides) according to the ratio of their mass and charge**

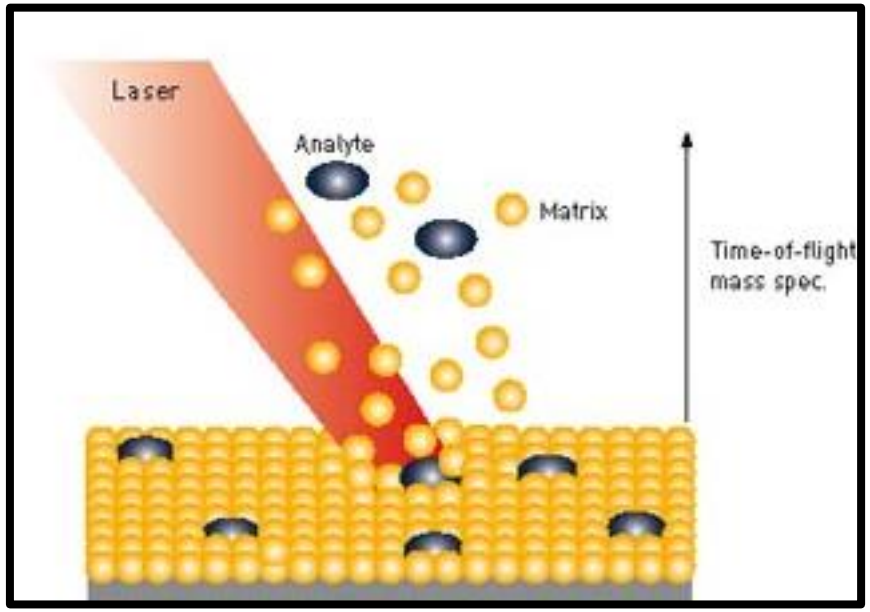
- **The molecule is first ionized using the MALDI or ESI (electrospray ionization) method**
- **The resulting ions are drawn into the analyzer by the electric field, where they are divided according to the ratio of mass and charge**
- **Computerized data processing follows**

Princip of MALDI-TOF

matrix-assisted laser desorption ionization time-of-flight

- **variant of mass spectrometry**
- **peptides are ionized and the mass-to-charge ratio is determined based on the time-of-flight to the detector**

- **M_w is calculated and is specific for each amino acid**



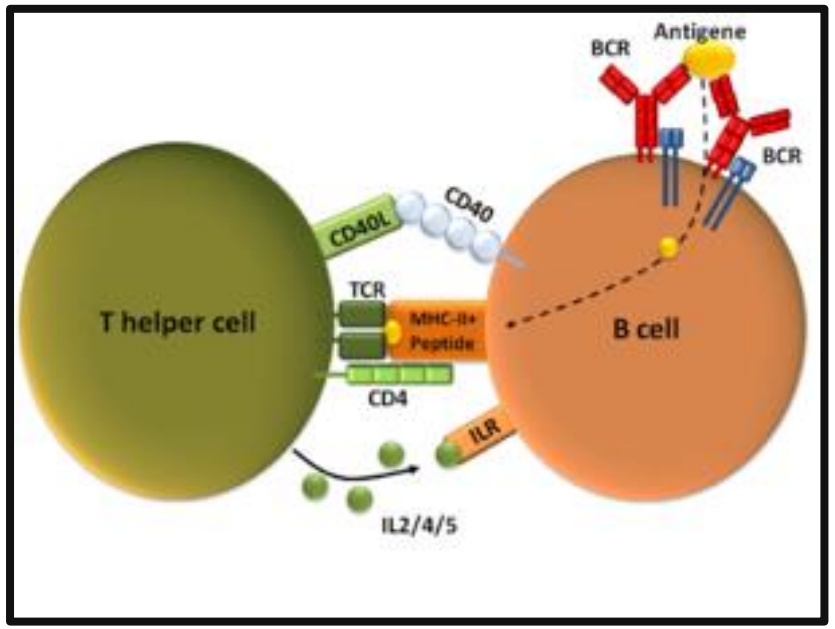
Advantages and disadvantages

1) There is no need to sequence the proteins
2) Just knowing the molecular weight is enough

1) Cannot analyze multimeric proteins
2) The AA sequence should be known

Antibodies as a basic detection and identification tool

- Used to study translation results
- They arise as a reaction of the organism to an antigen



The region of the antigen recognized by the antibody is called an epitope

Epitopes

Linear

- Antibodies bind to them regardless of conformation
- They recognize, for example, denatured proteins

Conformational

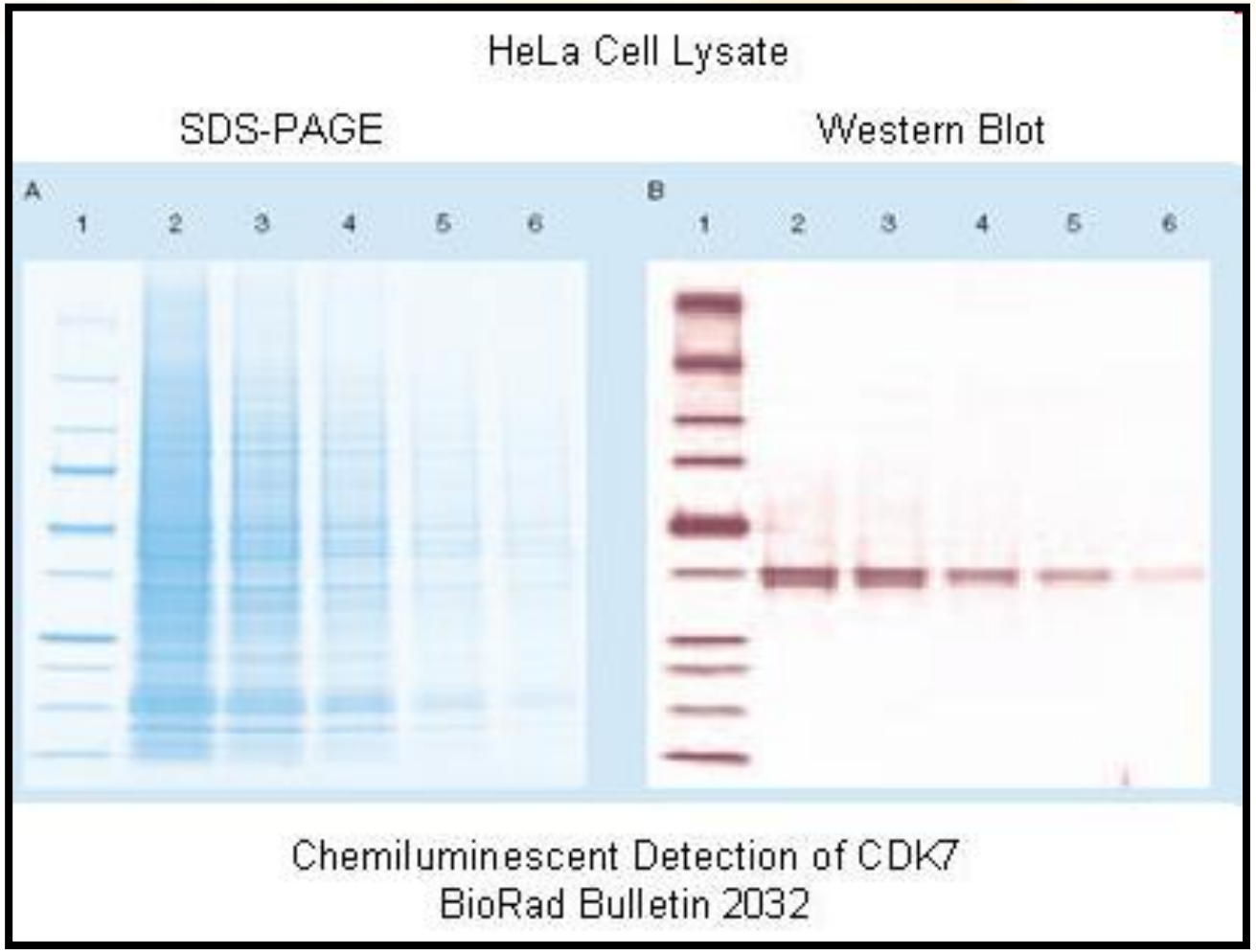
- Antibodies bind to them depending on the way the polypeptide chain is folded
- Antibodies specific for conformational epitopes will only react with proteins in the native conformation

Western blotting

Imunoblotting

- Identification of polypeptides by antibodies after separation on denaturing PAGE (alkaline buffer = proteins acquire a negative charge
- Recognition does not take place in the gel, but after transferring the polypeptides to a membrane

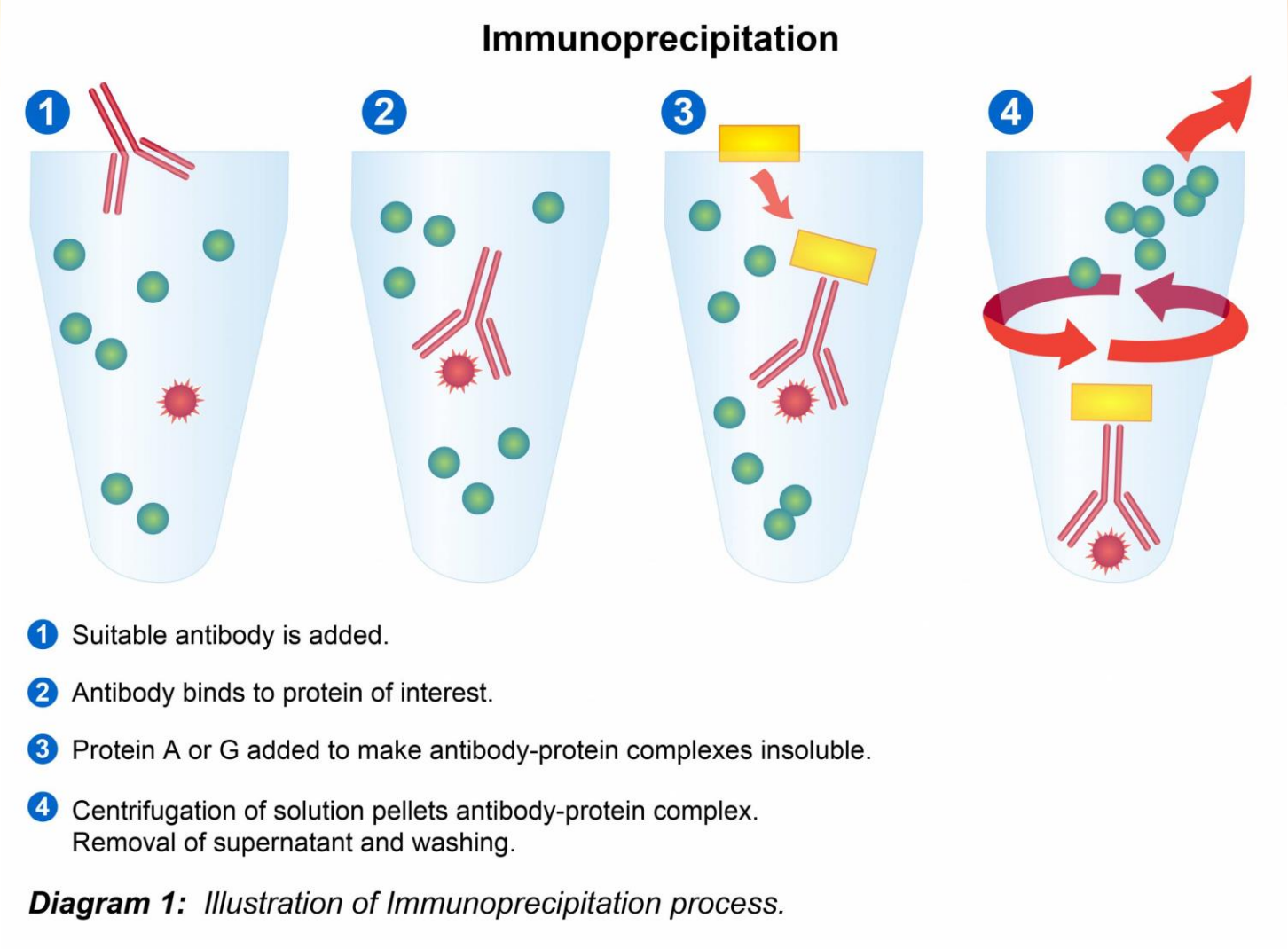
Immunoblotting result



Chemiluminescent Detection of CDK7
BioRad Bulletin 2032

Immunoprecipitation

- It is used to isolate specific proteins from protein mixtures using antibodies
- It is used to study interactions between proteins



https://www.leinco.com/immunoprecipitation/