UNIVERSITY OF CAMBRIDGE

Protein Purification 1: Lysis and Affinity Purification

Day 3: Wednesday 21nd March

Today's talk

- Bacterial protein expression
  - Harvesting cultures
  - Lysing your cells and clearing the lysate
- Mammalian protein expression
  - Harvesting cells/supernatant
  - Lysis and clearing the lysate/supernatant
- Affinity chromatography
  - Immobilised Metal Affinity Chromatography (IMAC)
  - Glutathione S-transferase purification
  - Biotin/Streptavidin and Strep-II/Strep-Tactin
  - Other options
- Engineered proteolysis
  - 3C (PreScission), TEV, Thrombin, SUMO

Harvesting your bacterial culture

- For cytoplasmic proteins, harvest by centrifugation
  - 5000 × g for 15 min
- Decant off the supernatant
- Scrape pellets into a beaker for immediate processing, or a tube for storage
- Pellets can be stored for months at -20°C for a few months, or at -70°C for years and years and years…

Images: Stephen Graham (CC BY 4.0)

Bacterial cell lysis

- Mechanical
  - Cell disruptor
  - Sonicator
  - Freeze-thaw
- Chemical
  - Detergent-based

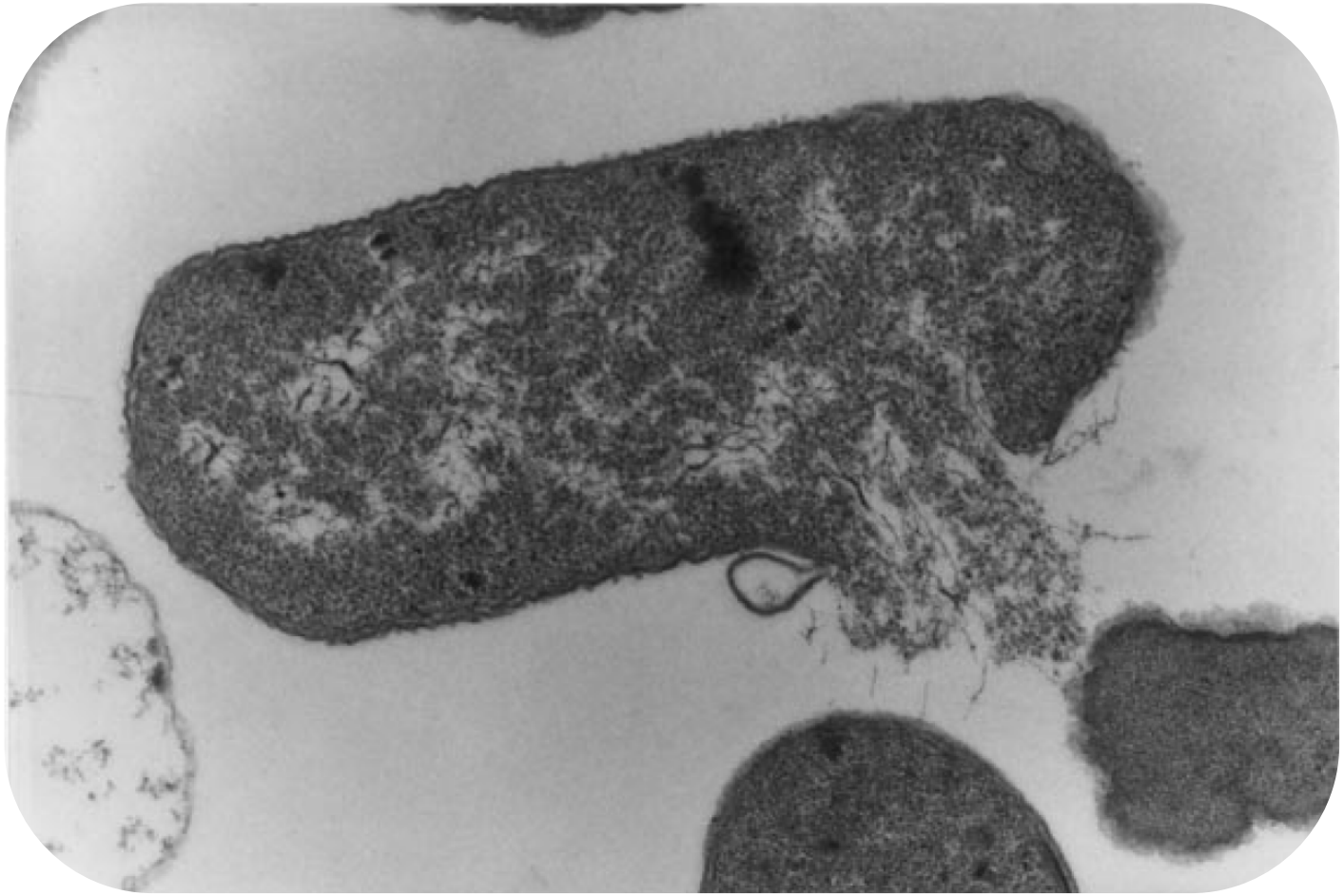
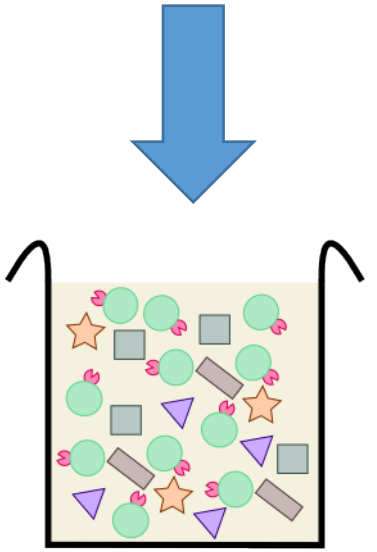


Image: Witte et al. (2006) *FEMS Microbiology Letters* 164: 159-67

Preparing to mechanically lyse cells

- Cells need to be resuspended in a **lysis buffer**
  - Important that you control the **pH** and **[salt]**
  - Often will include **reducing agents** and **protease inhibitors***
    - *EDTA-free for His-tagged proteins
  - I often include a tiny bit of detergent (0.05% or less)
  - Some proteins like glycerol to be present
  - Lysozyme can also help (especially for non-pLysS cells)
- Lysis buffer should generally be kept **cold**
  - Bacteria have proteases – even expression strains that lack OmpT and Lon
  - Proteases: very active at 37°C, poorly active at 4°C
  - Keep your lysate on ice until the affinity capture and wash to minimise proteolysis of your protein



Images: Stephen Graham (CC BY 4.0)

Preparing to mechanically lyse cells

- Important that cells are **thoroughly** resuspended for efficient lysis
  - No lumps!
- I resuspend cells on a stirring plate in the cold room using a magnetic flea (takes about 15-30 minutes)
  - Can be faster if you pipette up and down
- I lyse up to ~10 g of cell pellet per 50 mL of lysis buffer
  - Main aim is to have a smooth solution with no lumps!

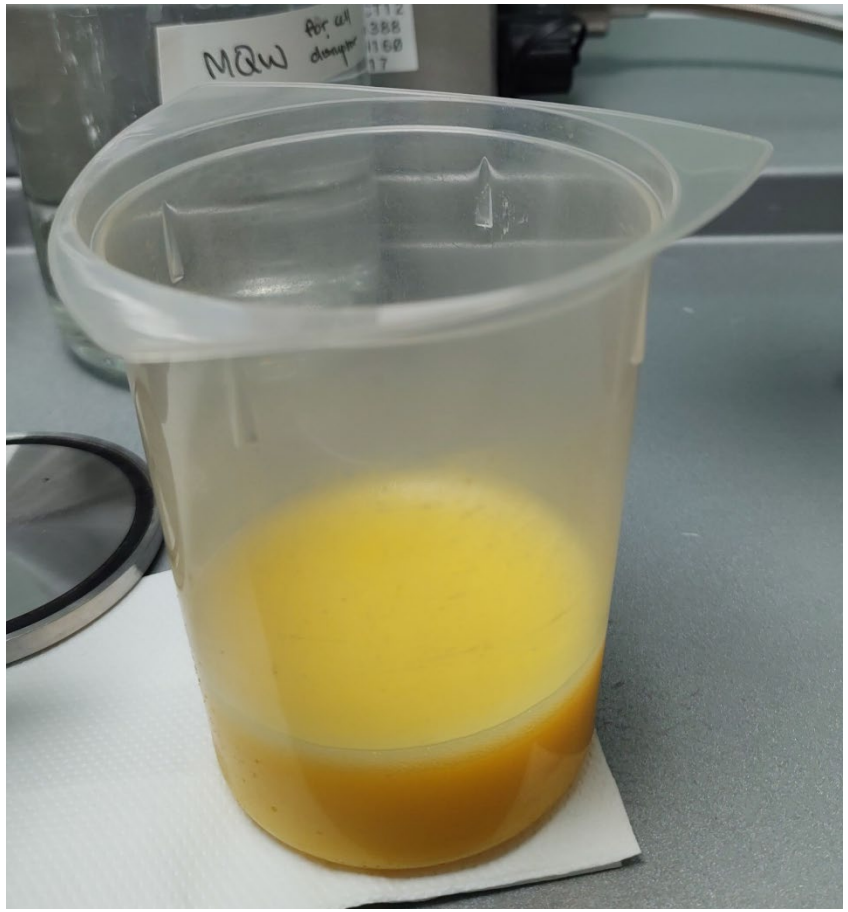


Image: Stephen Graham (CC BY 4.0)

Mechanical lysis: Cell disruptor

- Lyses cells via a combination of pressure, shear force and impact
- Sample is passed through an 100 µm diamond aperture at >20,000 psi
  - Significant shear force to disrupt sample, plus pressure change from high to low pressure
- Jet of liquid hits target and is disrupted by kinetic impact
- Consistent >90% lysis
- Some sample heating
  - Water-cooled jacket to cool samples

Images: Stephen Graham (CC BY 4.0)

Mechanical lysis: French Press or Pressure Homogeniser

- French press:
  - Similar to cell disruptor, large piston generates pressure to force cell suspension through narrow orifice
- Pressure Homogeniser (Emulsiflex)
  - Uses pressurised gas to force sample through a narrow orifice
- Both generate shear force to lyse cells
  - Very efficient cell lysis with some sample heating

Top Image: Glen Mills (https://www.glenmills.com/product-category/cell-disruption-and-culture/cell-disruption/french-press/)

Bottom Image: Avestin (https://www.avestin.com/emulsiflex-c5.htm)

Mechanical lysis: Sonication

- Use ultrasonic waves to disrupt cell membrane
  - Also very efficient at shearing DNA
- Causes localised sample heating
  - Need to sonicate as short bursts, preferably with sample on ice
- Ten cycles of 30s (with 30s rest) at 8 µm amplitude gives very efficient cell lysis
  - You might need to optimise this
- Sonicators can permanently damage your hearing - **You must wear ear protection!!**

Image: Stephen Graham (CC BY 4.0)

Chemical and other methods of cell lysis

- Freeze-thawing bacterial cells can cause significant cell lysis
- Hen egg white lysozyme can degrade the peptidoglycan of the bacterial cell wall, promoting lysis
- Detergents can be used to lyse bacterial cells
  - 1% TWEEN-20
- Combining all three can give reasonably efficient lysis (especially for small-scale tests)
  - But detergents and multiple freeze/thaws can also disrupt protein folding, lowering yield
- Proprietary cell lysis cocktails exist
  - Expensive for large/frequent protein preps

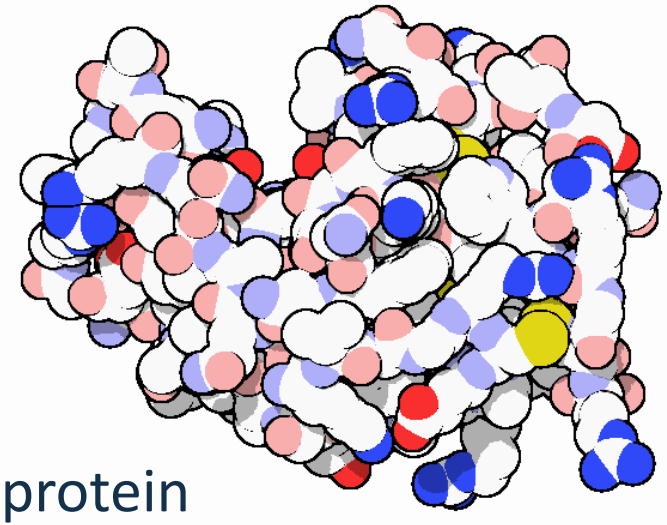


Image: RCSB PDB (https://pdb101.rcsb.org/motm/9)

Clearing cell lysates

- Remove chromosomal DNA, membranes and insoluble proteins
- Centrifuge at >20,000×g for ≥30 min
  - I routinely use 40,000×g for 30 min
- Pellet should be relatively small (<10% of total volume)
  - Large pellets are caused by inefficient lysis or presence of **inclusion bodies**
- Keep samples cold
- Reserve sample of cleared cell lysate for SDS-PAG

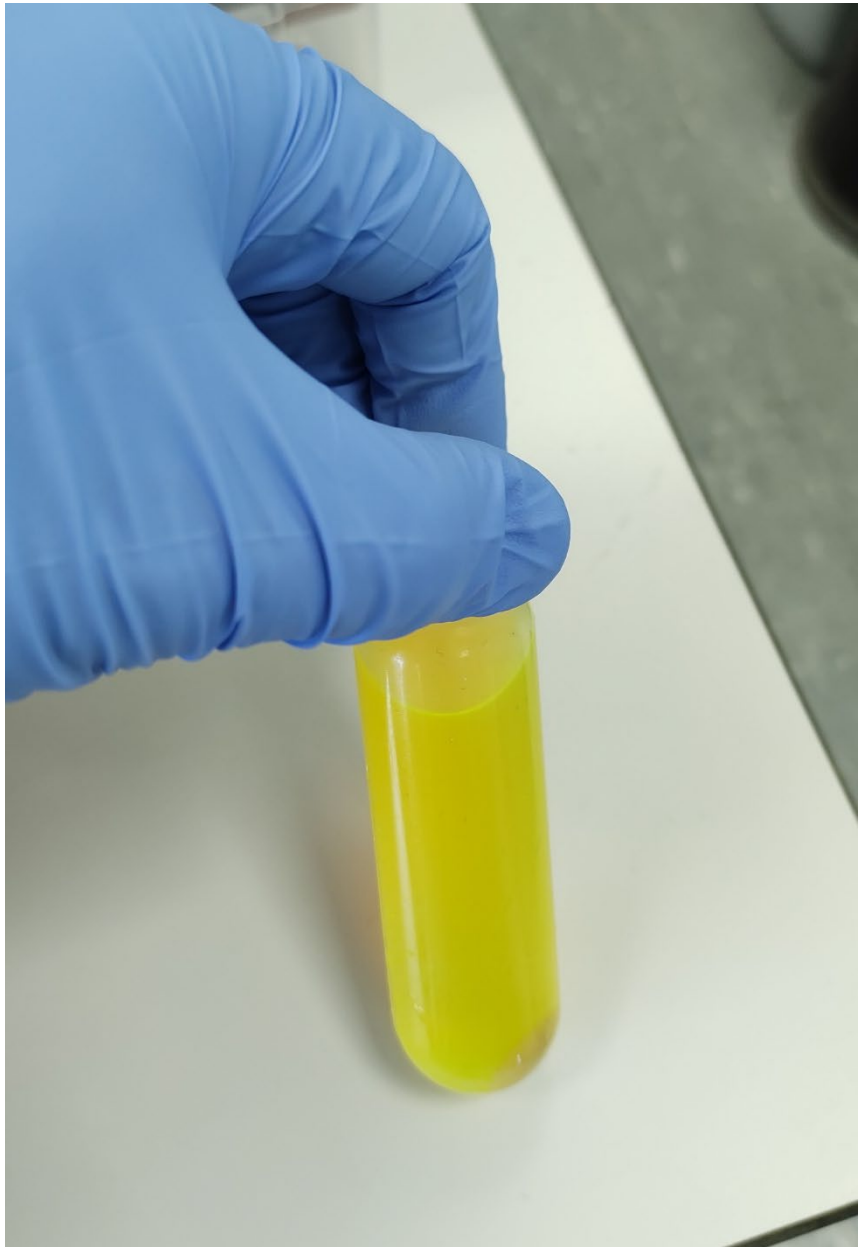


Image: Stephen Graham (CC BY 4.0)

Periplasmic bacterial expression

- Aim to liberate protein in periplasm of bacteria
  - Lyse outer cell wall but not inner cell wall
- Incubate with a hypertonic buffer (500 mM sucrose) to swell cells
- Transfer to hypotonic buffer (125 mM sucrose) to lyse periplasm
- Centrifuge to precipitate cell pellet
- Filter supernatant containing periplasmic proteins

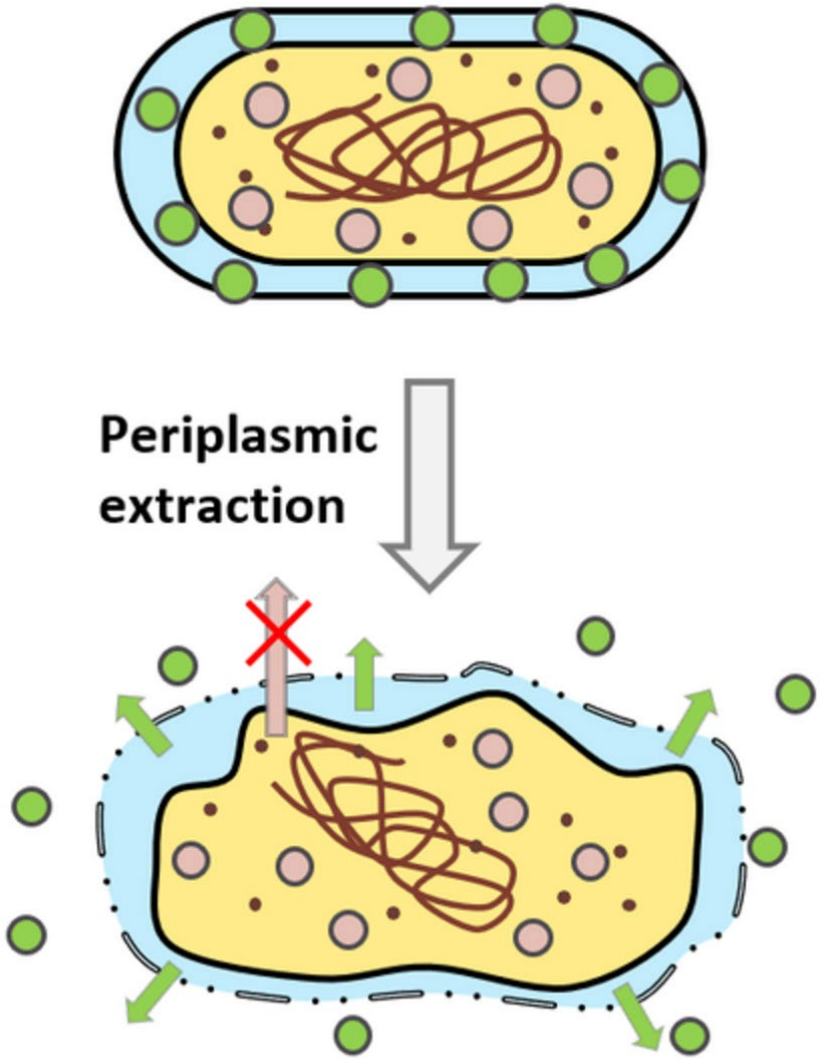


Image adapted from Schimek et al. (2020) *Biotechnology Progress* 36: e2999

Mammalian cell lysis (cytoplasmic proteins)

- Mammalian cells are much larger and more fragile than bacterial cells
  - Harvest at 200×g for 5 min to prevent premature lysis
- Very easy to lyse
  - **Passage through a 23G needle six times**
  - Osmotic shock in hypotonic buffer (10 mM Tris pH 7.5)
  - Incubation with detergent (1% Triton X-100)
  - Dounce homogeniser
- Clarify lysate by centrifugation (40,000×g, 30 min)

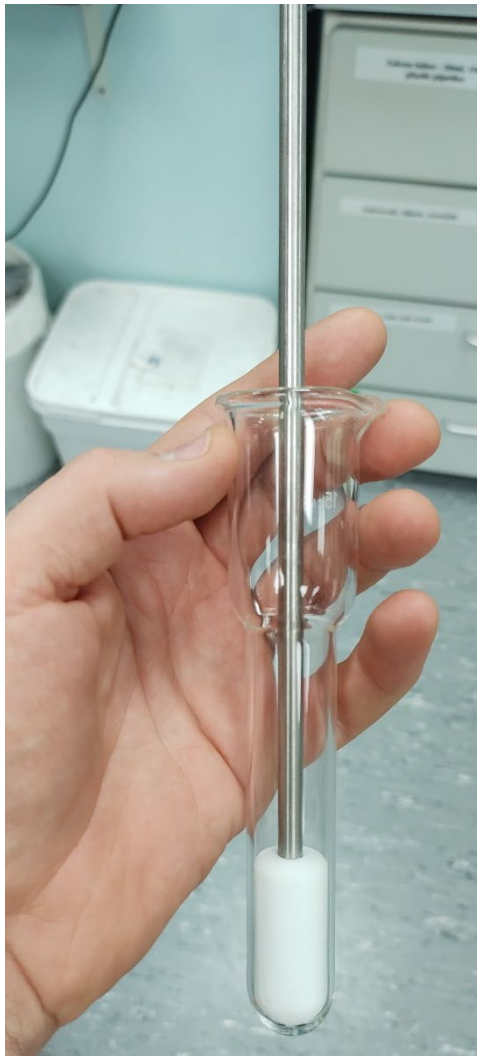


Image: Stephen Graham (CC BY 4.0)

Preparing mammalian cell supernatants

- Spin out cells
  (200×g for 10 min)
- Clarify lysate
  (40,000×g for 30 min)
- 0.2 µm filter if storing for a long time
  - Some secreted mammalian proteins like antibodies can be stored for years and years at 4°C…

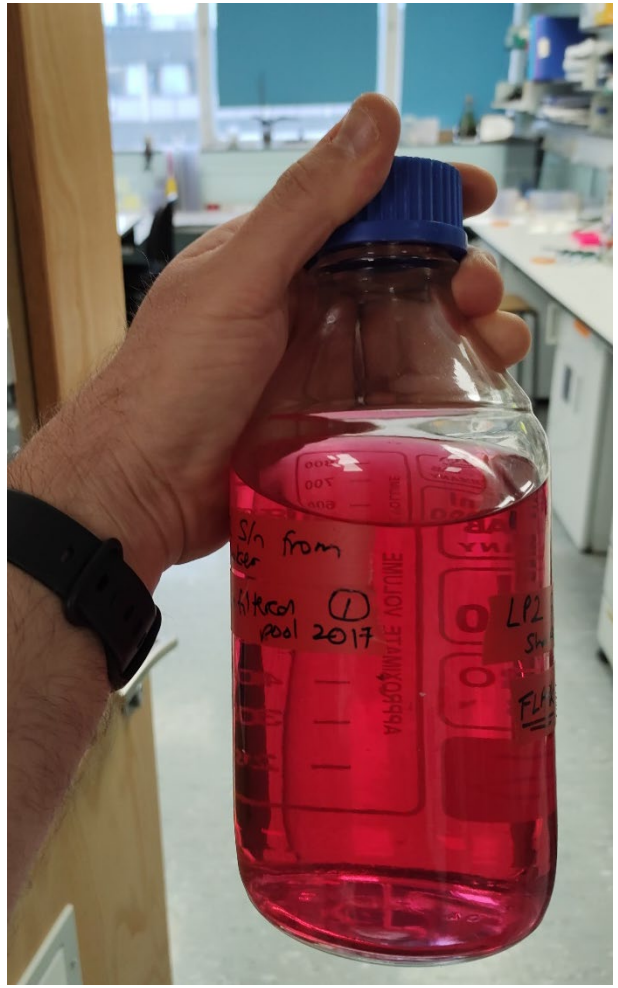


Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Selective resin (beads) that can capture your protein
- Separates your protein from the vast majority of other cellular proteins
  - Including **proteases**
- Concentrates your protein ready for further purification steps, if required (tomorrow's seminar)

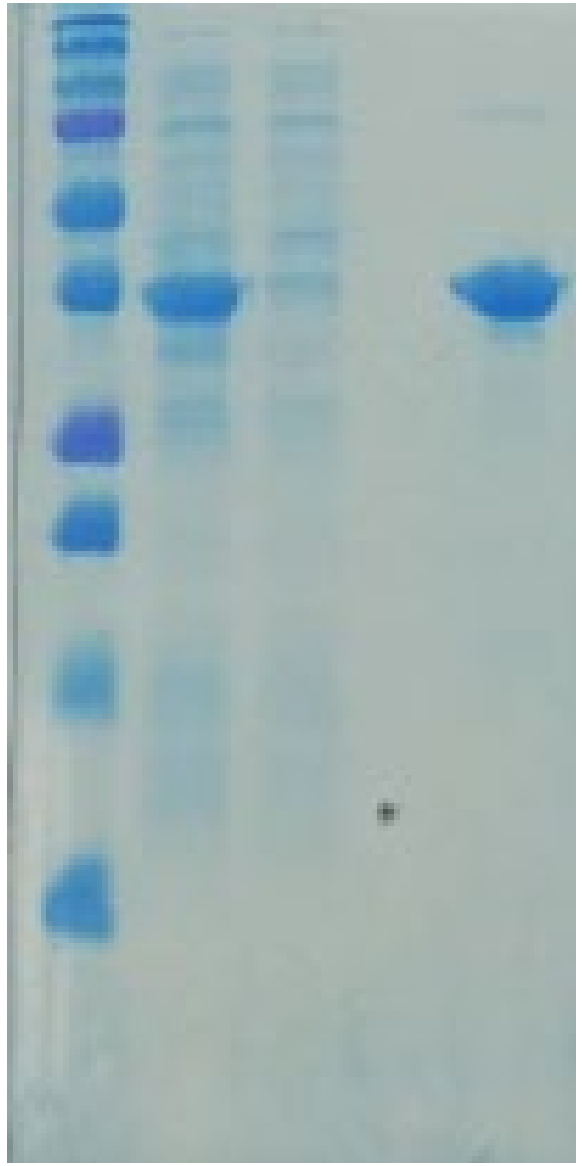


Image: Stephen Graham and Giulia Gallo (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)

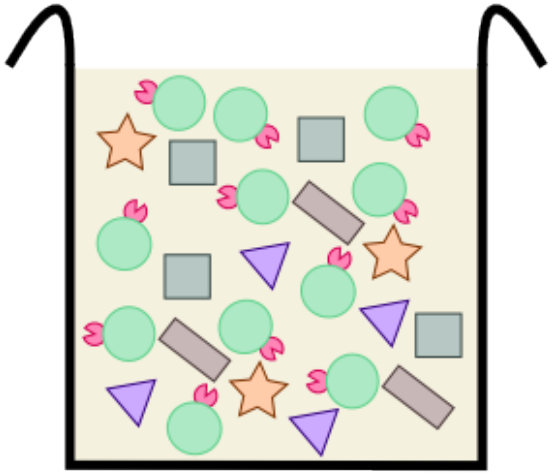


Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin

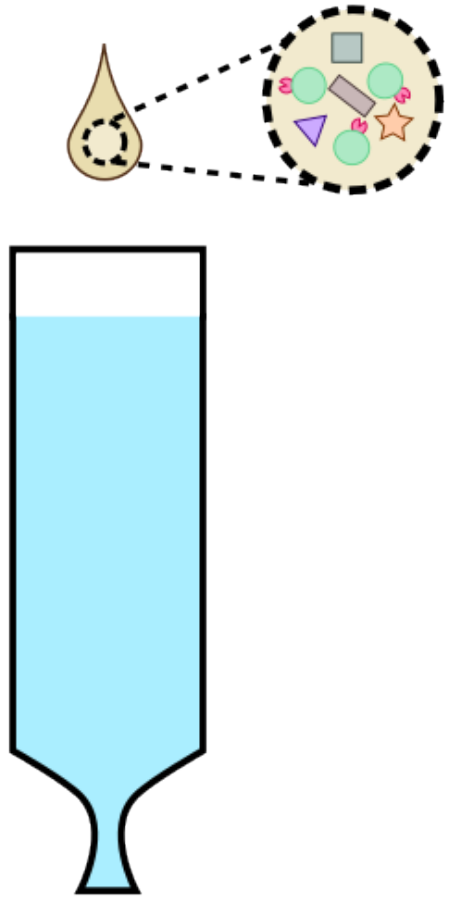


Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

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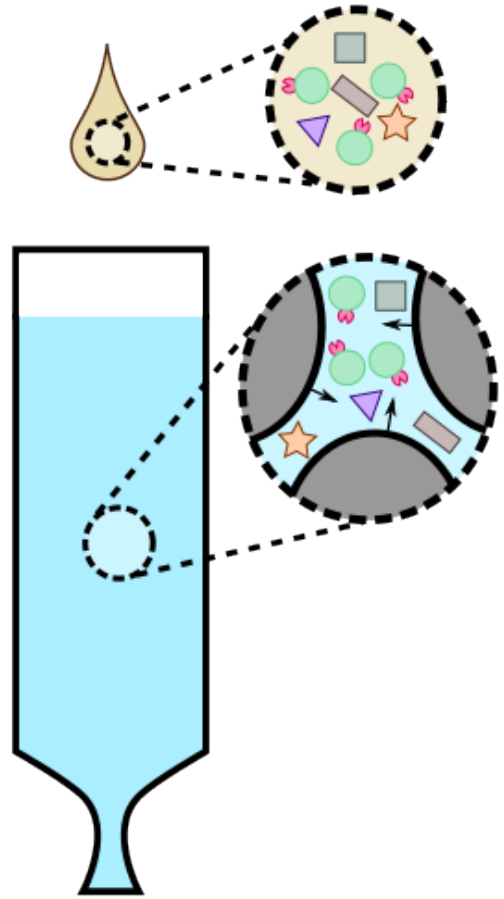

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin
- Selective resin will capture tagged protein

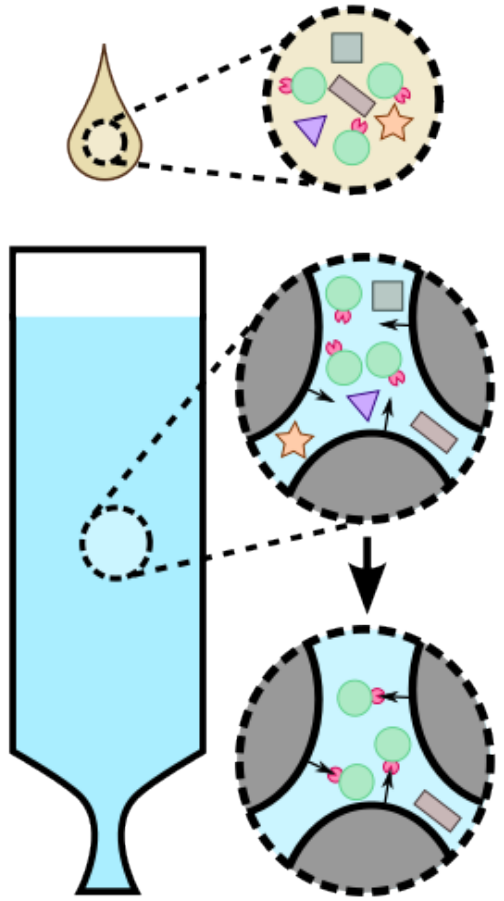

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin
- Selective resin will capture tagged protein
- Other proteins will **flow through** column

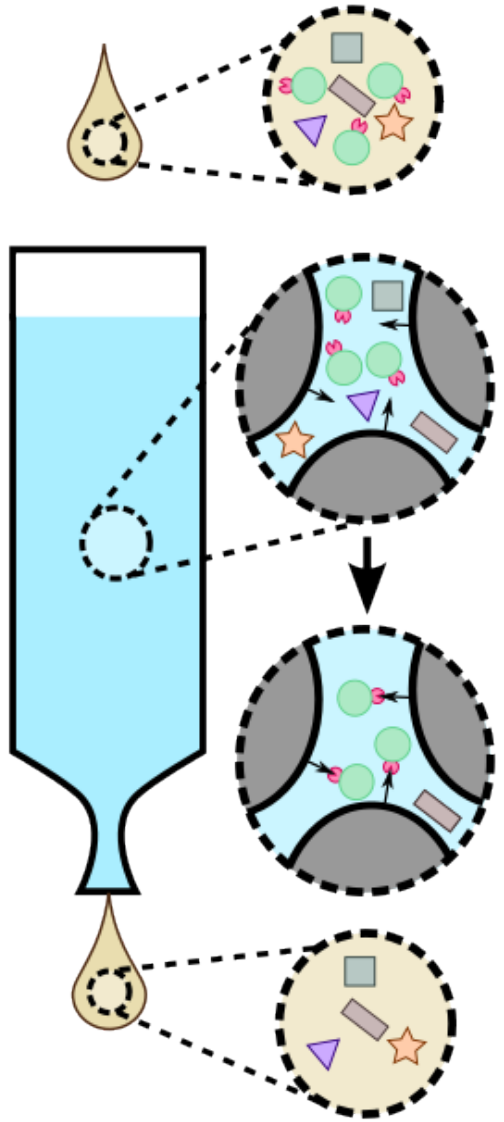

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin
- Selective resin will capture tagged protein
- Other proteins will **flow through** column
- Wash column with **wash buffer** to remove residual unbound protein

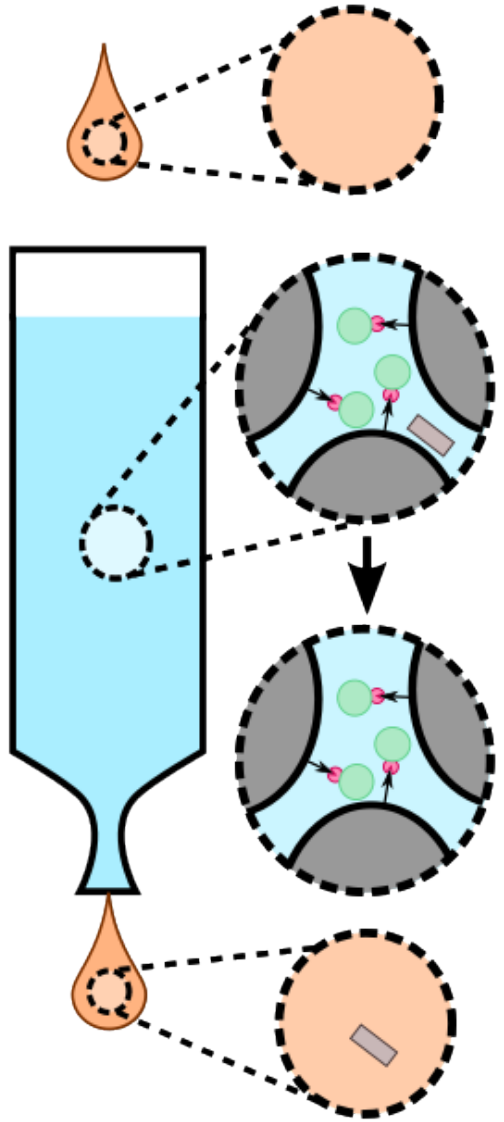

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin
- Selective resin will capture tagged protein
- Other proteins will **flow through** column
- Wash column with **wash buffer** to remove residual unbound protein
- Apply **elution buffer**
  - Contains molecule that competes with tag:column interaction

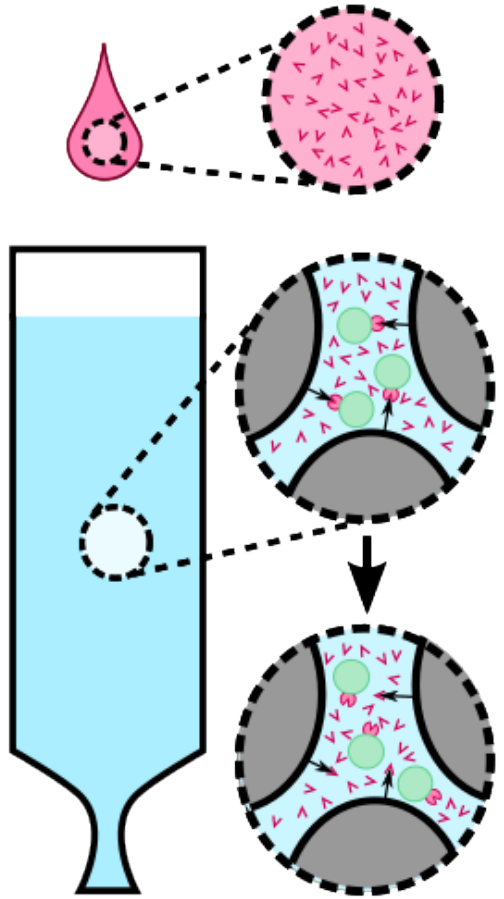

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography

- Start with clarified cell lysate (or supernatant)
- Apply to selective resin
- Selective resin will capture tagged protein
- Other proteins will **flow through** column
- Wash column with **wash buffer** to remove residual unbound protein
- Apply **elution buffer**
  - Contains molecule that competes with tag:column interaction
- Protein is eluted in elution buffer

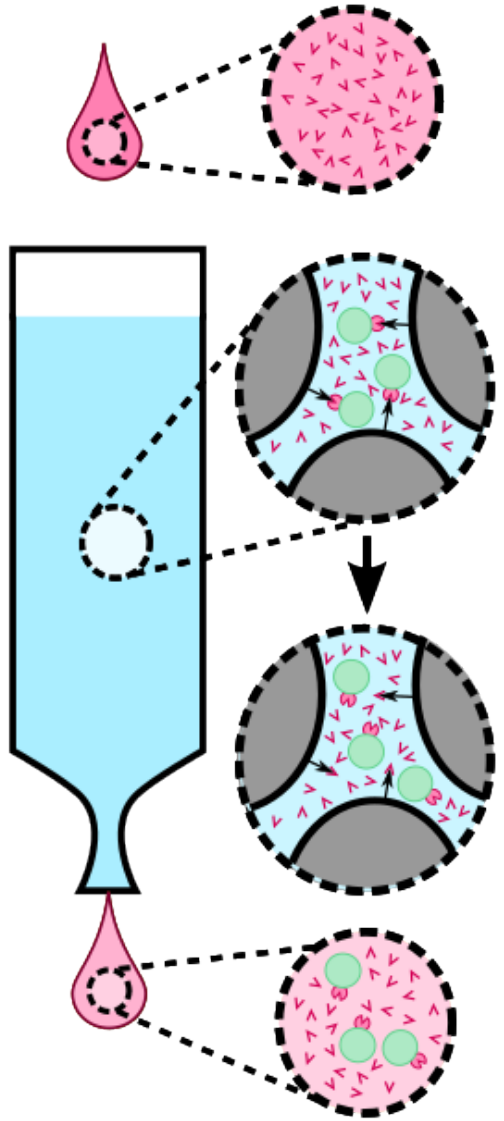

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography elution

- Elution buffer can be:
  - Molecule that mimics the resin:
    competes with resin for binding to the tag

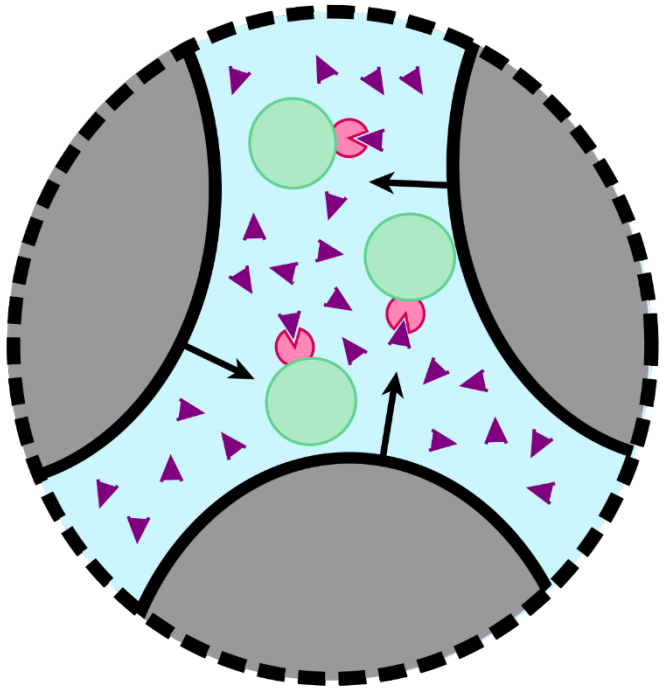

Image: Stephen Graham (CC BY 4.0)

Affinity chromatography elution

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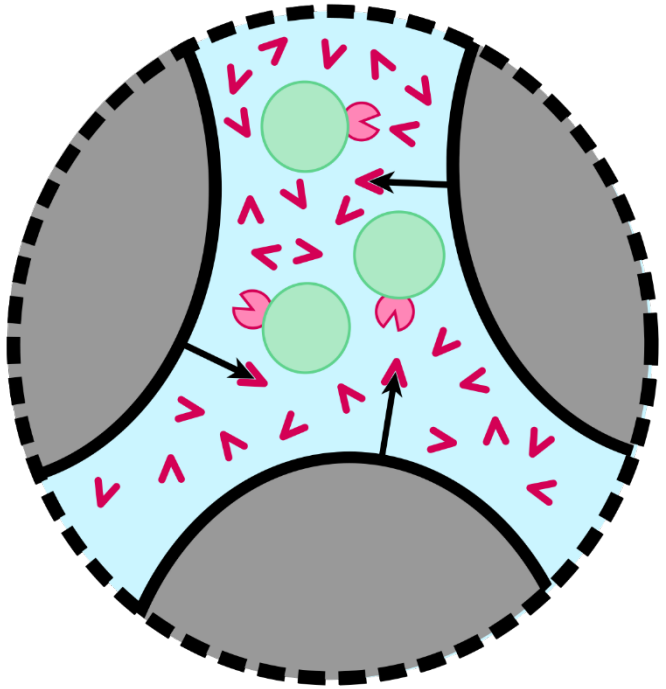


Image: Stephen Graham (CC BY 4.0)

Affinity chromatography elution

- Elution buffer can be:
  - Molecule that mimics the resin:
    competes with resin for binding to the tag
  - Molecule that mimics the tag:
    competes with tag for binding to the resin
- You can also elute protein by using a protease that cleaves between the protein and the tag
  - Perform *on column* proteolysis using engineered proteases and cleavage sites
  - Protein is eluted
  - You can then then regenerate column using small molecules as above

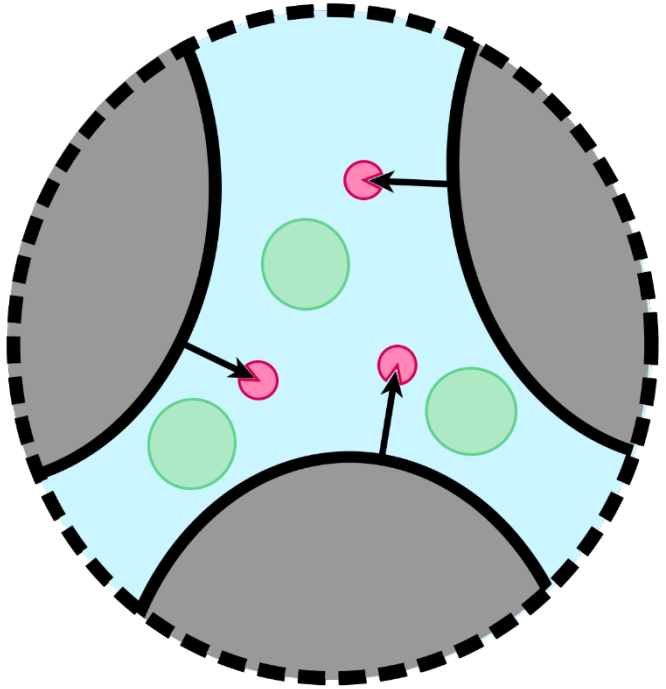


Image: Stephen Graham (CC BY 4.0)

Immobilised Metal Affinity Chromatography (IMAC)

- Exploits the affinity of histidine side chains for metal ions
- Encode a short polyhistidine tag at the N or C terminus of your protein
  - Usually His_6

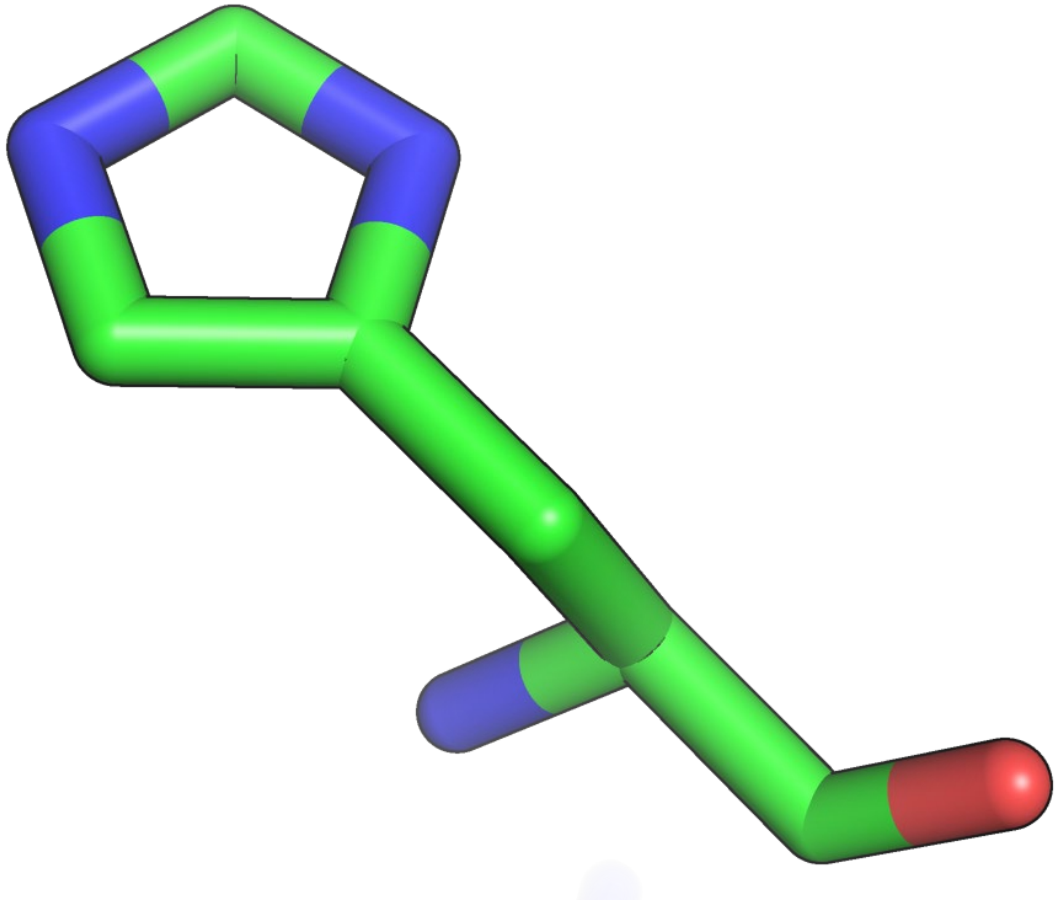


Image: Stephen Graham (CC BY 4.0)

Immobilised Metal Affinity Chromatography (IMAC)

- Exploits the affinity of histidine side chains for metal ions
- Encode a short polyhistidine tag at the N or C terminus of your protein
  - Usually His_6
- Resin has a metal chelating group
  - E.g. Nitrilotriacetic acid (NTA)

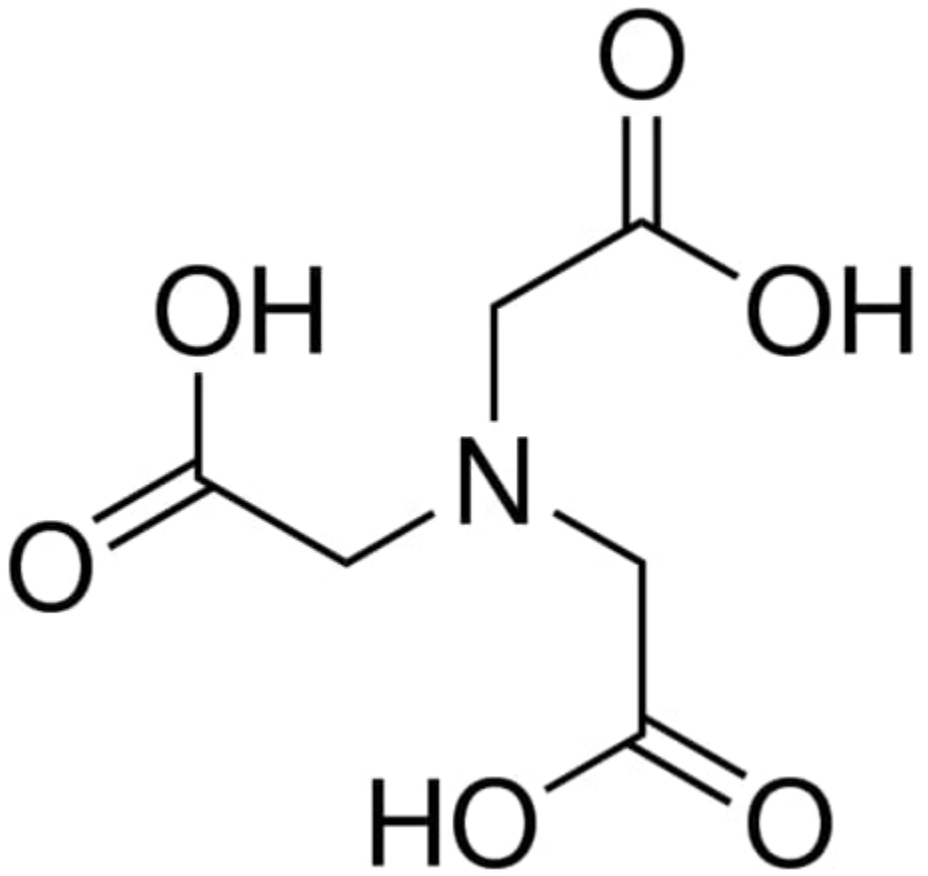


Image: Merck

Immobilised Metal Affinity Chromatography (IMAC)

- Exploits the affinity of histidine side chains for metal ions
- Encode a short polyhistidine tag at the N or C terminus of your protein
  - Usually His_6
- Resin has a metal chelating group
  - E.g. Nitrilotriacetic acid (NTA)
- Most common metal ion is Ni^{2+}
  - Co^{2+} is also used

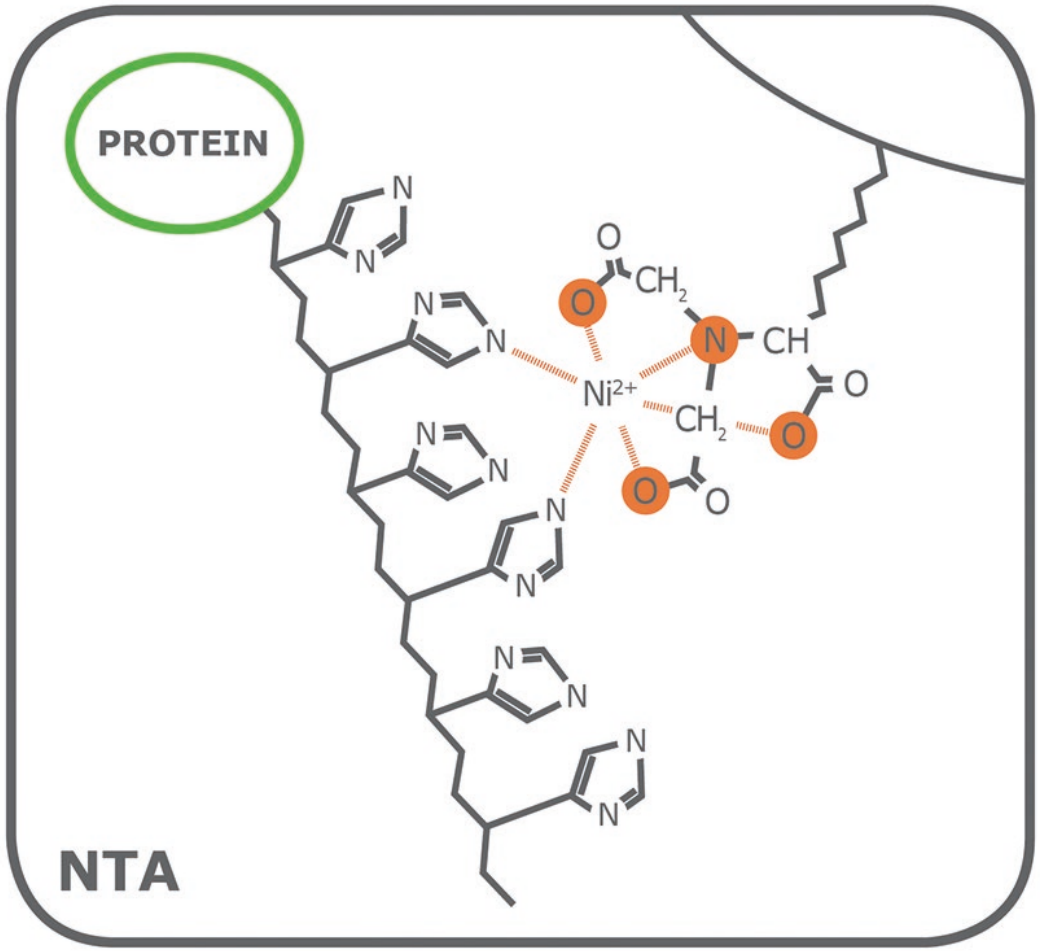
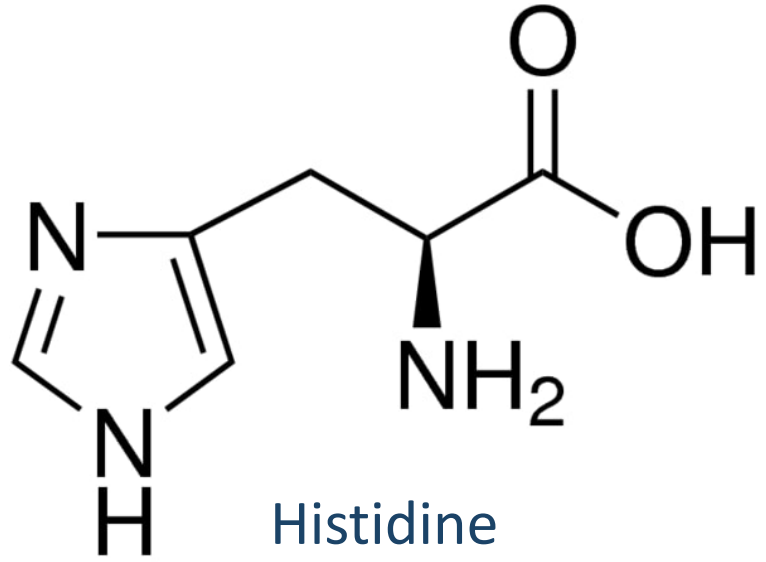


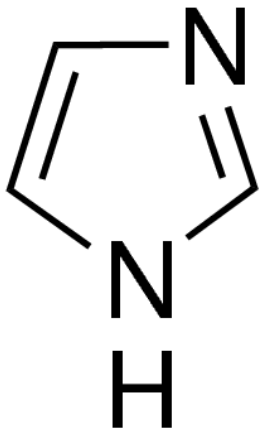
Image: Cube Biotech

Ni-NTA IMAC

- Protein is eluted using imidazole
  - >200 mM imidazole
  - Competes with His side chain for binding Ni-NTA
- NiNTA columns have (relatively) high non-specific binding
  - Use low concentration (10-20 mM) imidazole in wash buffers
  - Use high salt in wash buffers
- Imidazole is a chaotropic agent
  - Unfolds proteins
  - Remove as soon as possible after elution (dialysis, etc)



Histidine



Imidazole

Images: Merck

Glutathione S-transferase (GST) affinity chromatography

- Glutathione S-transferase (GST) is a small (26 kDa) protein that catalyses conjugation of reduced glutathione (GSH) to other chemicals
  - Detoxification of foreign substances
- GST binds to GSH with high affinity
- GST tags are appended to the N or C termini of proteins
  - Can increase solubility of tagged protein
  - Efficiently captured by GSH conjugated to a resin

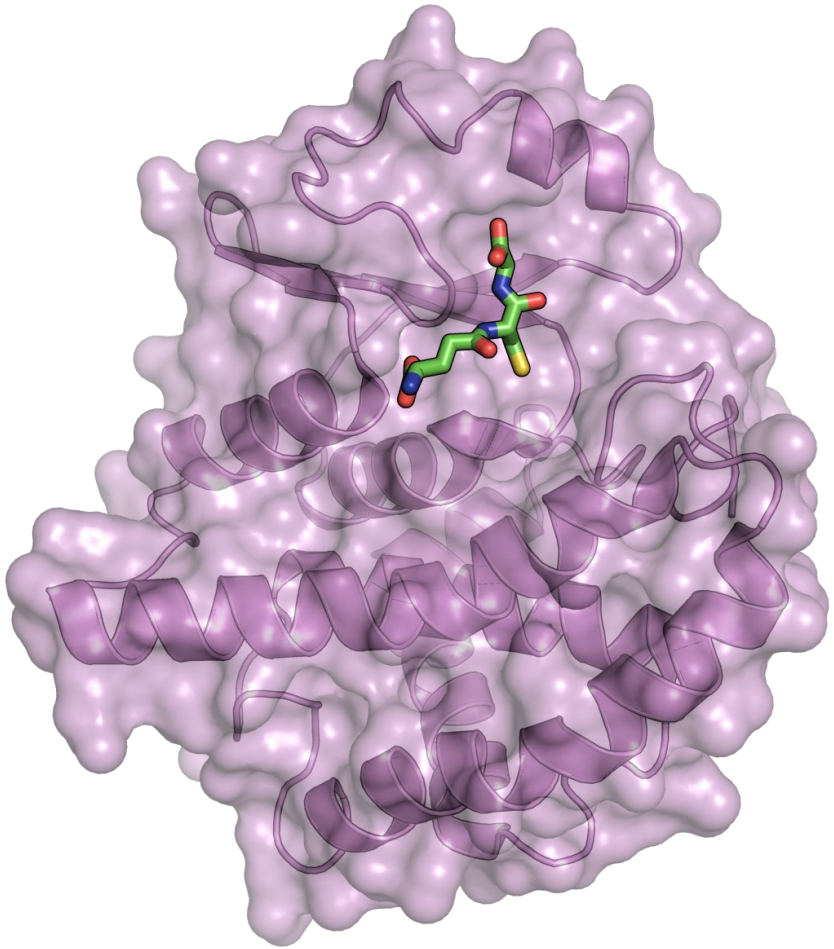
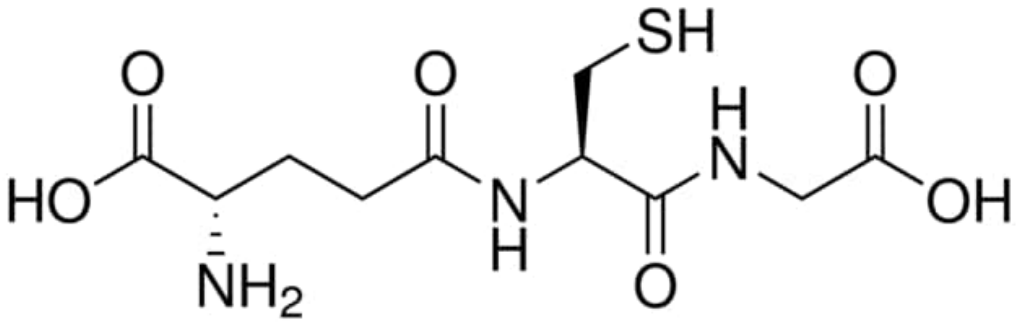


Image: PDB 1UA5 [Kursula et al (2005) *Protein Pept Lett* 12, 709-12], Stephen Graham (CC BY 4.0)

GST affinity chromatography

- Highly specific binding
  - Low background
- High affinity interaction
  - Allows extensive washing
- Elute with relatively mild conditions
  - 20 mM GSH in normal buffers
- Relatively slow binding kinetics
  - Need longer incubations for binding and elution



Reduced L-glutathione (GSH)

Note the isopeptide bond

Image: Merck

GST affinity chromatography

- Highly specific binding
  - Low background
- High affinity interaction
  - Allows extensive washing
- Elute with relatively mild conditions
  - 20 mM GSH in normal buffers
- Relatively slow binding kinetics
  - Need longer incubations for binding and elution
- Large tag that dimerises
  - Often removed for downstream applications

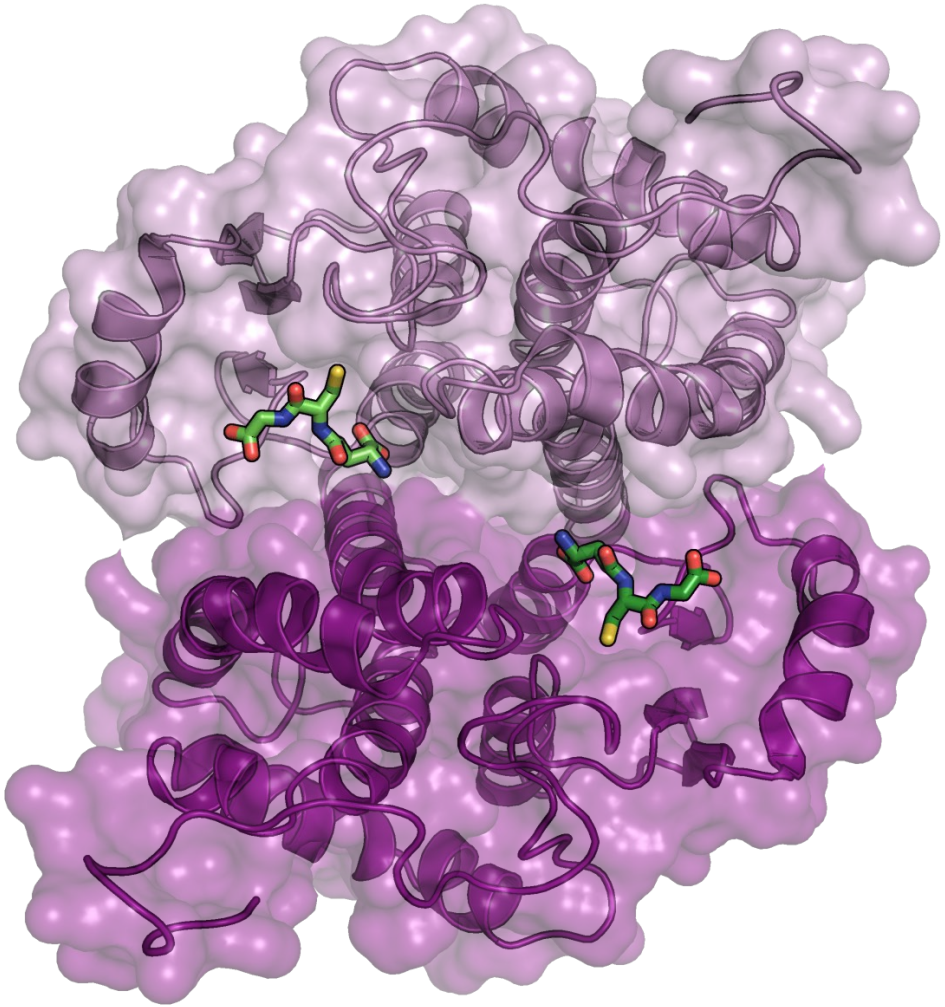


Image: PDB 1GNE [Lim et al (1994) *Protein Sci* 3, 2233-44], Stephen Graham (CC BY 4.0)

Streptavidin and biotin

- Biotin binding to streptavidin is one of tightest known non-covalent interactions
- Proteins can be covalently biotinylated if conjugated to a biotin acceptor peptide (BAP) sequence
- Can use streptavidin resin to purify proteins
  - Highly specific to biotin
- Some endogenously biotinylated proteins in *E. coli* will co-purify
- Interaction is so strong that elution is very difficult without denaturation

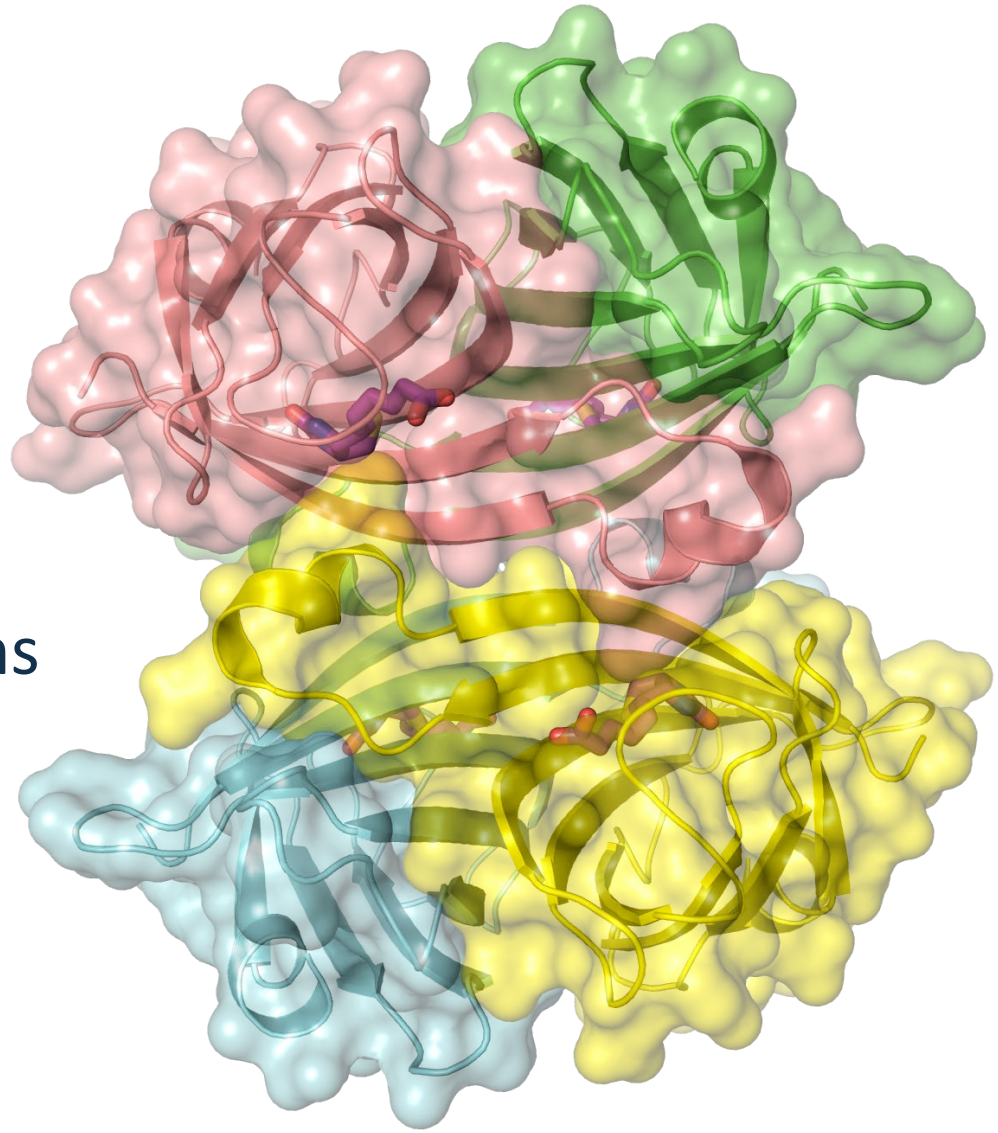


Image: PDB 1STP [Weber et al (1989) *Science* 243, 85-88], Stephen Graham (CC BY 4.0)

Strep-II tag and Strep-Tactin

- A short peptide sequence was identified with intrinsic affinity toward streptavidin
  - **Strep-II tag**: WSHPQFEK
- Streptavidin was engineered to have enhanced affinity for the Strep-II tag and reduced affinity for biotin
  - First generation was Strep-Tactin
  - Latest generation is Strep-Tactin XT
- Can use a twin Strep-II tag for avidity-enhanced binding

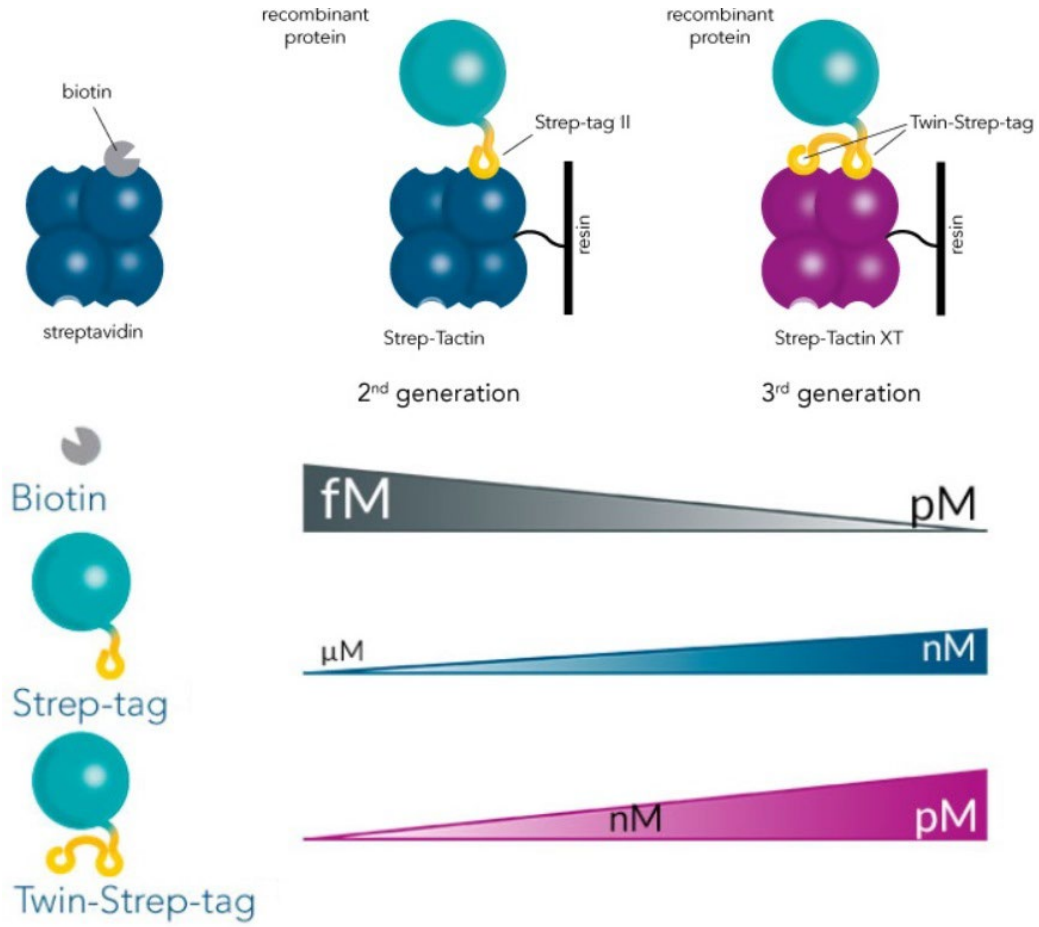


Image: IBA Lifesciences

Strep-Tactin XT

- Combines benefits of a short tag sequence with high specificity and high affinity binding
- Simple protein elution (biotin) and regeneration ($MgCl_2$)
- High binding capacity (5 mg/mL)
- Resin is quite expensive

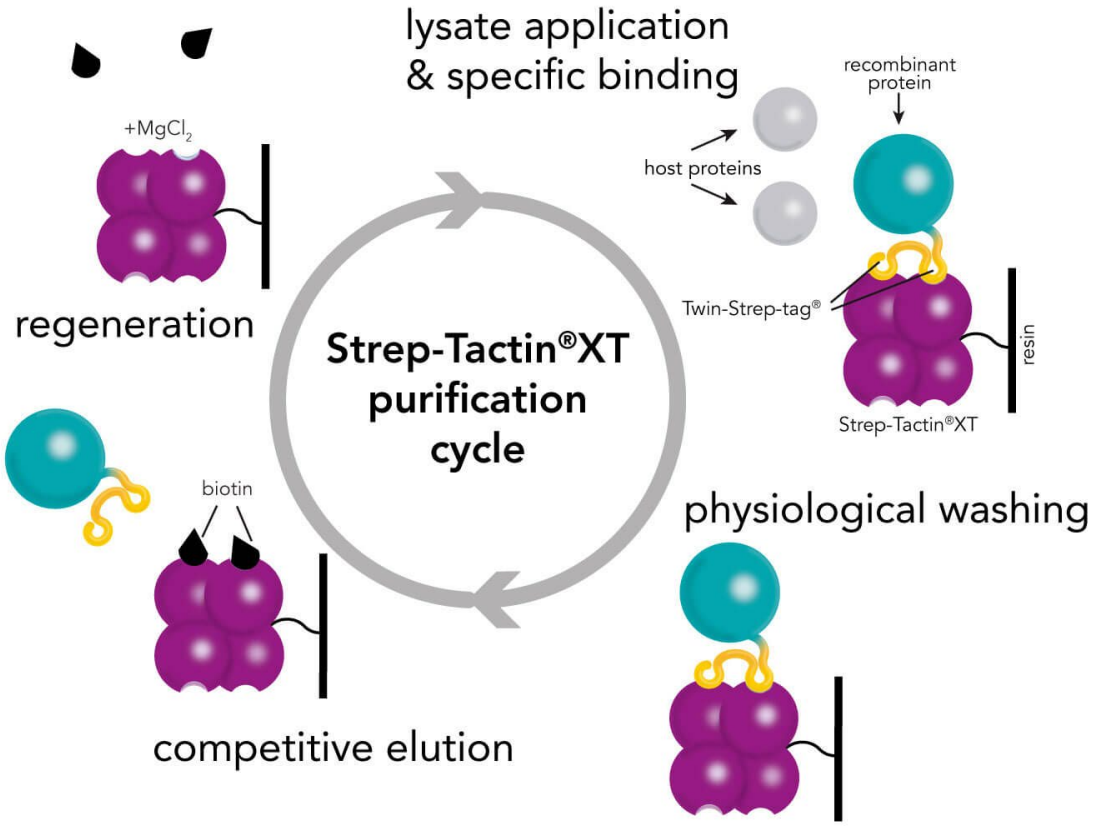


Image: IBA Lifesciences

Antibody affinity columns

- Antibodies/nanobodies combine high specificity and affinity
- Antibodies or nanobodies covalently coupled to agarose resin can be used as affinity columns
  - Antibodies against your protein of interest
  - Antibodies against an epitope tag appended to your protein (e.g. HA, FLAG)
- Elute using low pH or excess of the engineered epitope (synthetic peptide)
- Quite expensive resin and regeneration

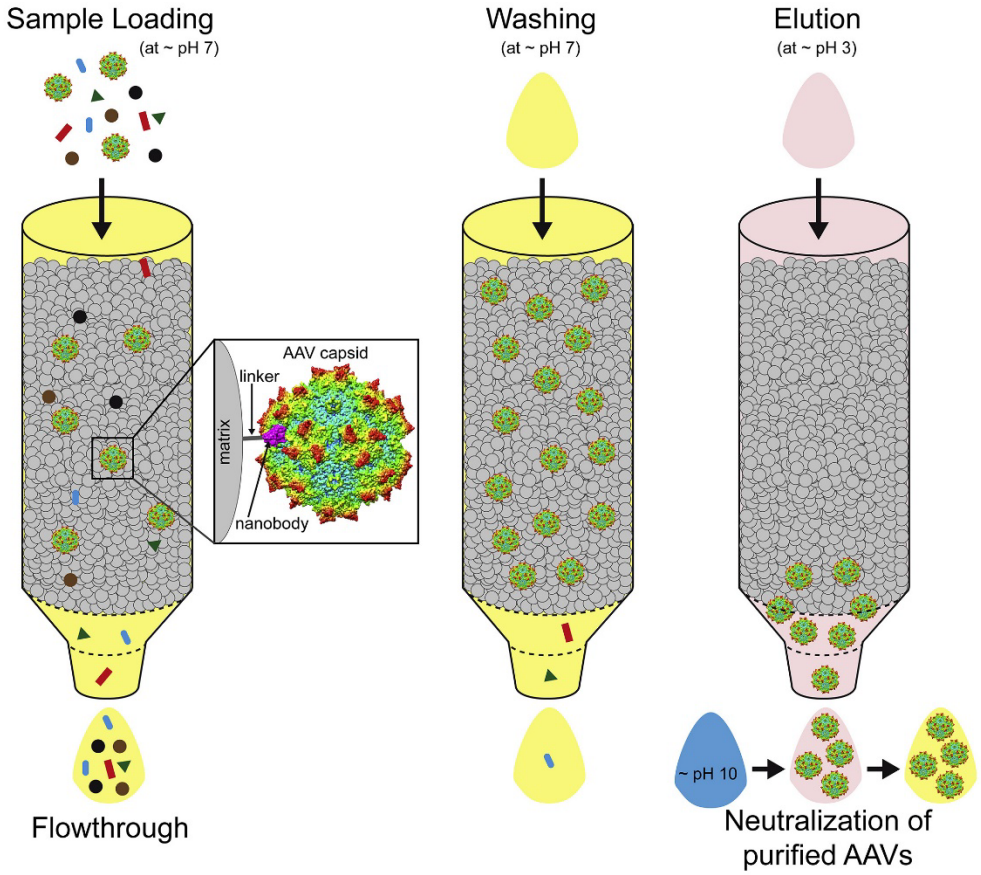


Image: Mietzsch et al. (2020) *Mol Ther Methods Clin Dev* 19, 362-373

Protein A, Protein G and Protein A/G

- Protein A and Protein G are antibody-binding proteins from *Staphylococcus aureus* and *Streptococcus* bacteria
- Bind the Fc (A) and Fc + Fab (G) domains of mammalian antibodies
  - Different affinities for different isotypes
- Protein A/G is a recombinant fusion of the IgG binding domains of proteins A and G
- Proteins A, G and A/G are excellent for antibody purification

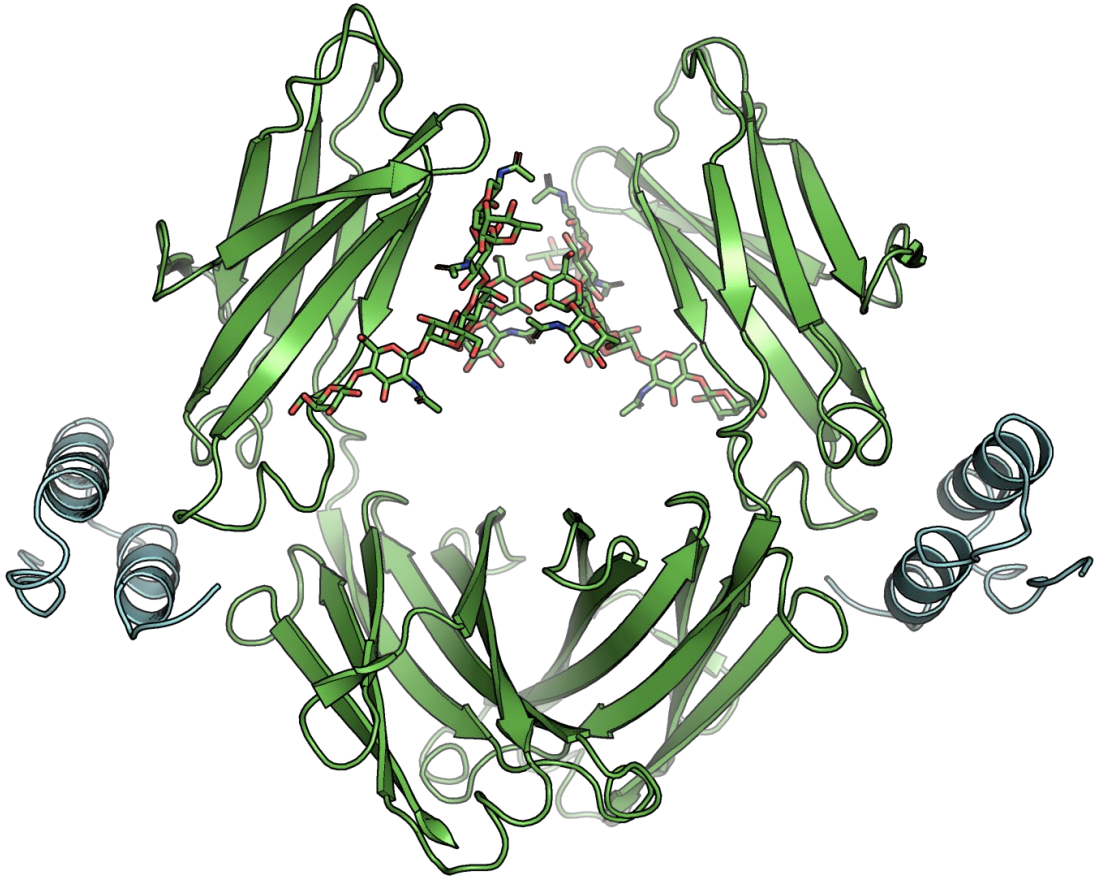


Image: PDB 1FC2 [Deisenhofer (1981) *Biochemistry* 20, 2361-2370], Stephen Graham (CC BY 4.0)

Protein A tags and IgG columns

- Protein A can be used as an affinity tag
  - May enhance solubility of proteins expressed via secretion from mammalian cells
- Can use IgG fused to agarose to capture tagged proteins
- Cleave protein off column using engineered protease
- Retained protein A can be eluted using low pH

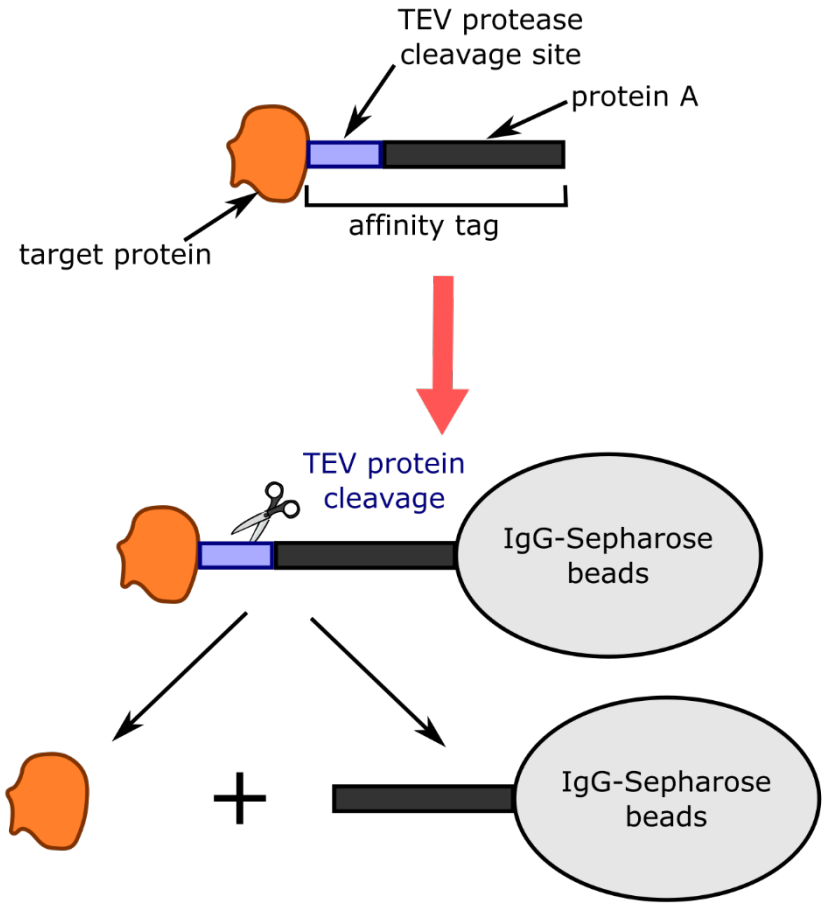


Image: Stephen Graham (CC BY 4.0), adapted from Wikimedia (Philippe Hupé – CC SA 3.0)

Which expression tag is for me?

Tag/Resin	Pros	Cons
His/NiNTA	Small tag High capacity resin (>10 mg/mL) Resin and imidazole are cheap	Higher non-specific binding Lower affinity (can ‘over-wash’) Elute with chaotropic chemical (imidazole)

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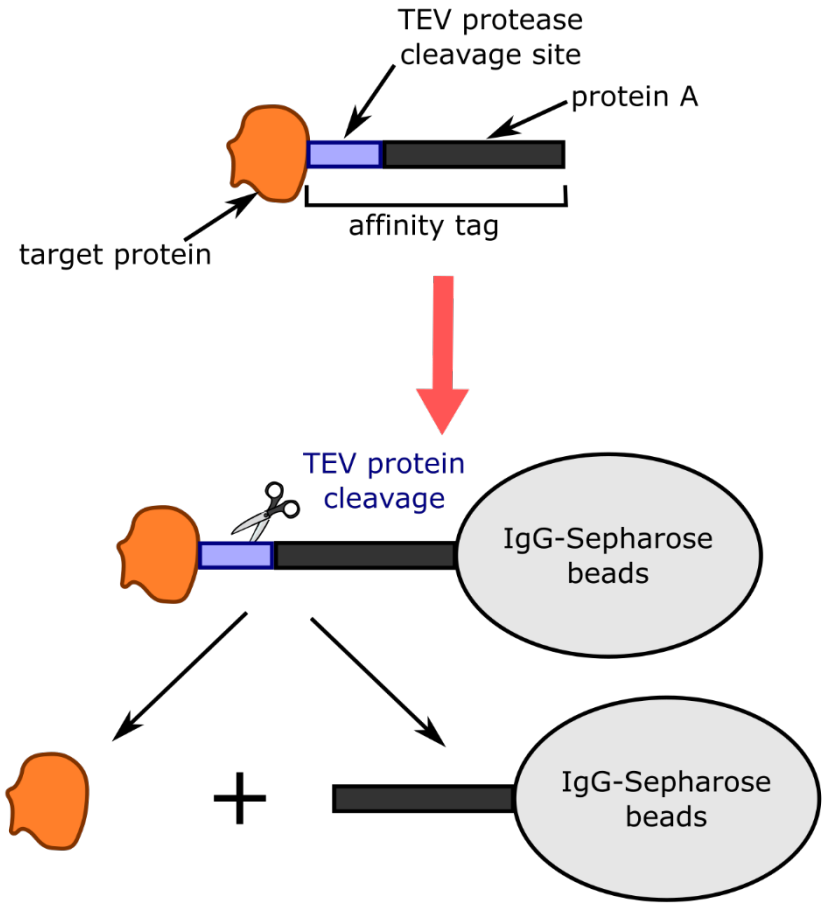
Biotin/Streptavidin	Super-high affinity	Super-high affinity
Antibody columns	Very specific	Expensive and lower capacity
Protein A and IgG columns	High affinity specific binding Protein A may solubilise proteins	Need to cleave protein off resin Slightly trickier resin storage and regeneration

Engineered proteolysis

- Non-specific proteolysis is A Bad Thing
- But proteolytic removal of tags can be helpful:
  - Elute protein from affinity resin without using harsh elution buffers (e.g. low pH)
  - Remove large purification tags that cause oligomerisation or could interfere with function (e.g. GST)
- Can design proteolysis sites into expression constructs and use specific purified proteases to mediate cleavage



3C (PreScission) protease

- Cysteine protease
  - Active under reducing conditions, inhibited by divalent cations (e.g. Zn^{2+})
- From human rhinovirus 14
  - Cleaves viral polyprotein
- Recognition sequence: LEVLFQ/GP
- Sold by Cytiva as “PreScission protease”
- Easy to express in *E. coli* and purify

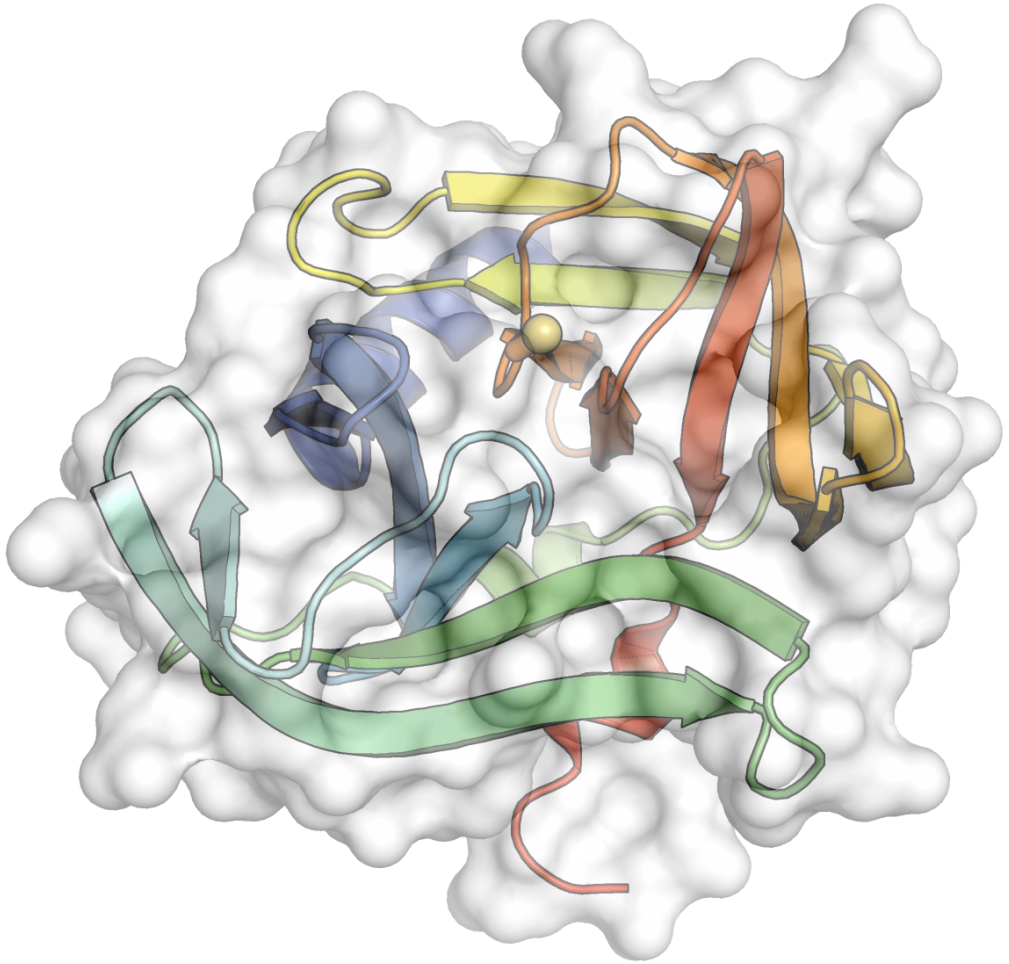


Image: PDB 2IN2 [Bjorndahl et al. (2007) *Biochemistry* 46, 12945-58], Stephen Graham (CC BY 4.0)

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  - We use GST fusion
  - Allows capture of protease and liberated GST after cleavage

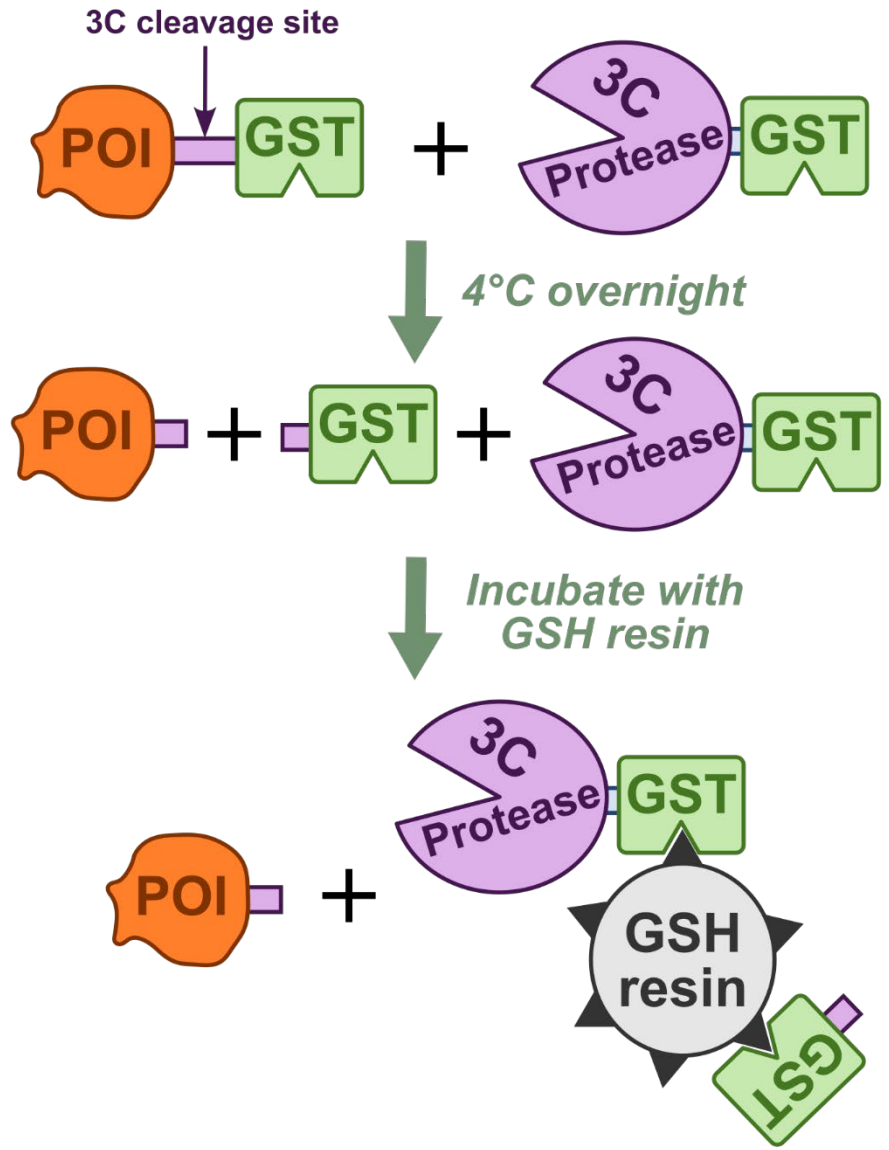

Image: Stephen Graham (CC BY 4.0)

TEV protease

- Cysteine protease
- From tobacco etch virus
  - Cleaves polyprotein
- Recognition sequence: ExxYxQS/G
  - x is any amino acid
- Wild-type TEV:
  - Undergoes autoproteolysis that reduces activity
  - Is poorly expressed in *E. coli* and poorly soluble
- Engineered mutants of TEV exist to overcome these issues
  - And are tagged for easy removal

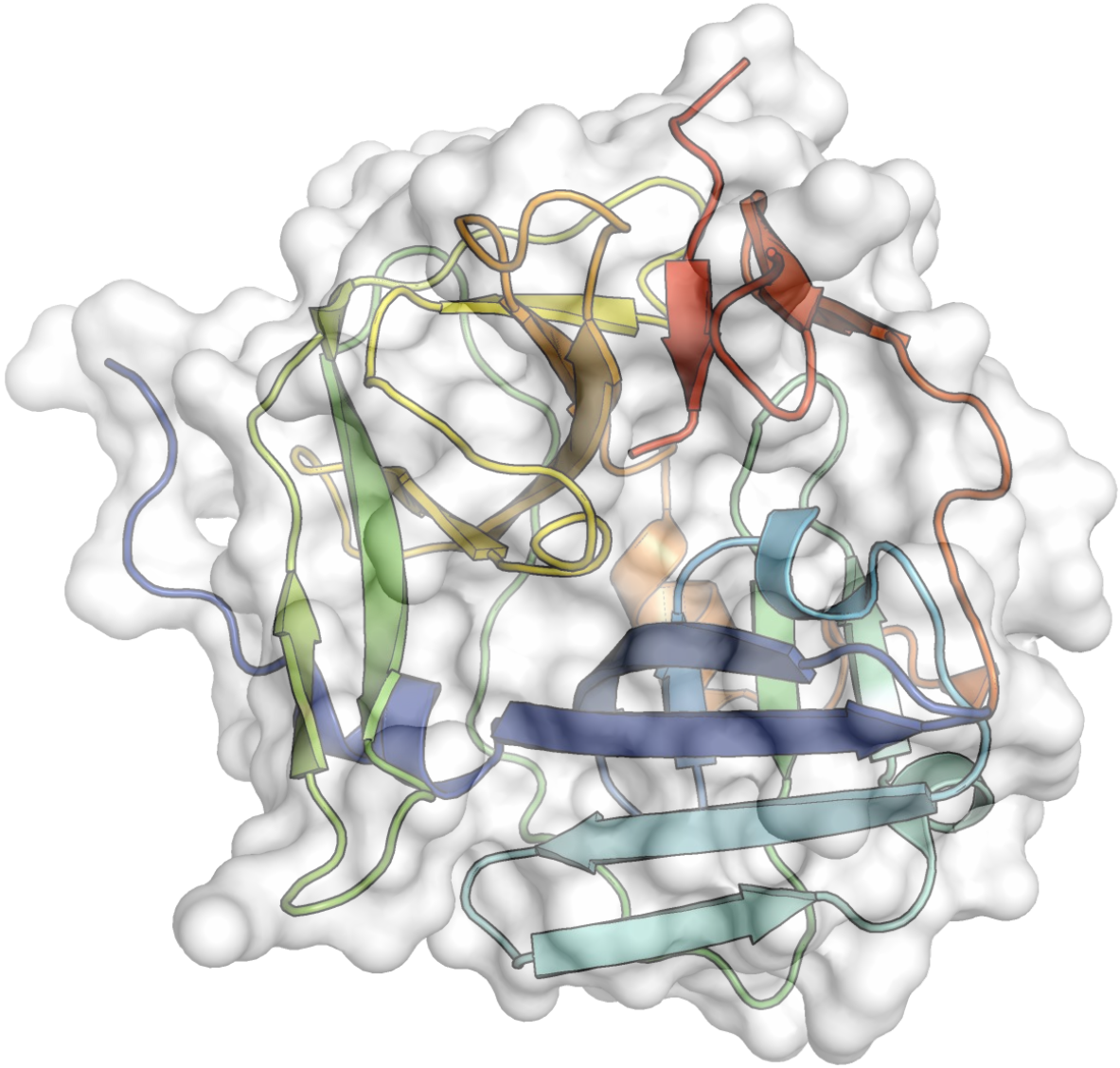

Image: PDB 1Q31 [Nunn et al. (2005) *J Mol Biol* 350, 145-155], Stephen Graham (CC BY 4.0)

Thrombin

- Serine protease
  - Inhibited by PMSF or AEBSF
- Acts as a blood coagulation factor
  - Cleaves fibrinogen to fibrin
  - Expressed as a zymogen (pro-enzyme) and activated via cleavage by factor Xa
- Recognition sequence: LVPR/GS
- Abundant in blood of mammals
  - Bovine thrombin can be purchased relatively cheaply
  - Purity varies between vendors…

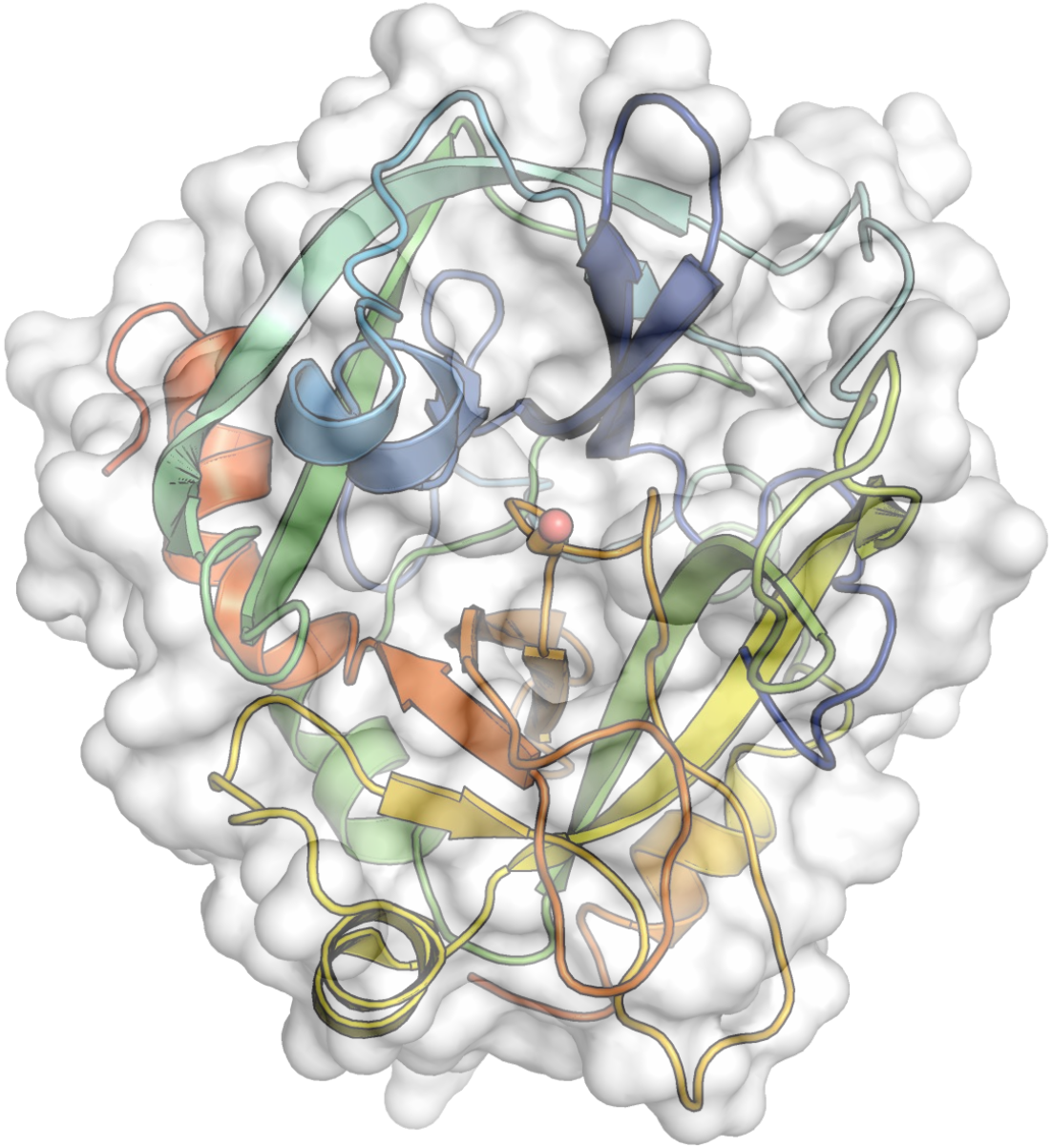

Image: PDB 1ETR [Brandstetter et al. (1992) *J Mol Biol* 226, 1085-1099], Stephen Graham (CC BY 4.0)

SUMO protease

- SUMO is a small ubiquitin-like protein
  - Covalently attached to eukaryotic proteins
  - Alters their subcellular localisation and activity
- SUMOylation can be rapidly reversed by deSUMOylases *in vivo* and *in vitro*
  - Recognise folded SUMO protein and cleave C-terminal sequence xGG/x
- N-terminal SUMO tags can enhance protein solubility
- Removed without a ‘scar’ using SUMO protease
  - Highly active SUMO protease from thermophilic bacteria *Chaetomium thermophilum*

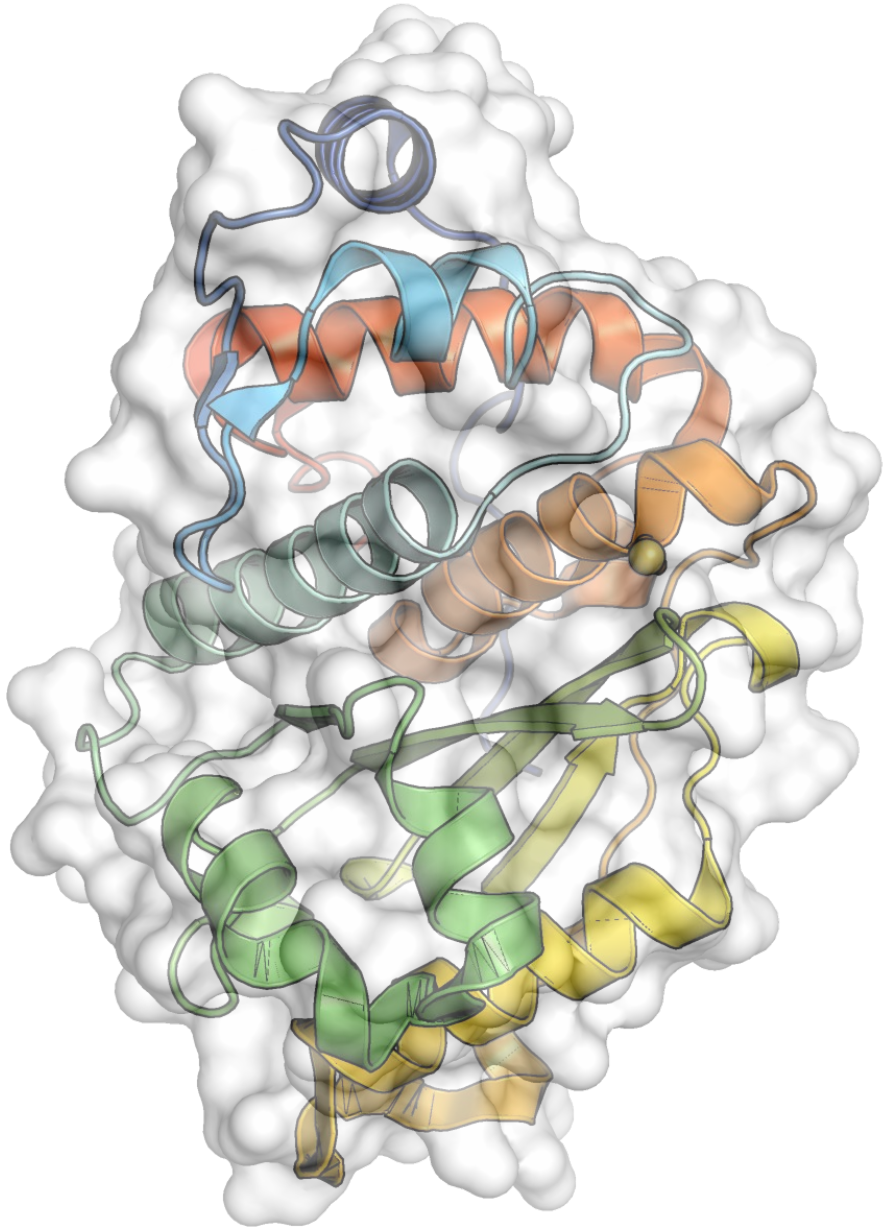

Image: PDB 6DG4 [Lau et al. (2018) *J Biol Chem* 293, 13224-33], Stephen Graham (CC BY 4.0)

Today's talk

- Bacterial protein expression
  - Harvesting cultures
  - Lysing your cells and clearing the lysate
- Mammalian protein expression
  - Harvesting cells/supernatant
  - Lysis and clearing the lysate/supernatant
- Affinity chromatography
  - Immobilised Metal Affinity Chromatography (IMAC)
  - Glutathione S-transferase purification
  - Biotin/Streptavidin and Strep-II/Strep-Tactin
  - Other options
- Engineered proteolysis
  - 3C (PreScission), TEV, Thrombin, SUMO

Tomorrow:

Beyond affinity capture: additional techniques to further purify your protein