UFABC

Bacharelado em Ciência & Tecnologia

Purificação de proteínas

PROTEÍNAS RECOMBINANTES

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Universidade Federal do ABC

Técnicas de purificação de proteínas

- Homogeneização
- Fracionamento (sulfato de amônio),
- Centrifugação
- Diálise

Técnicas de purificação de proteínas

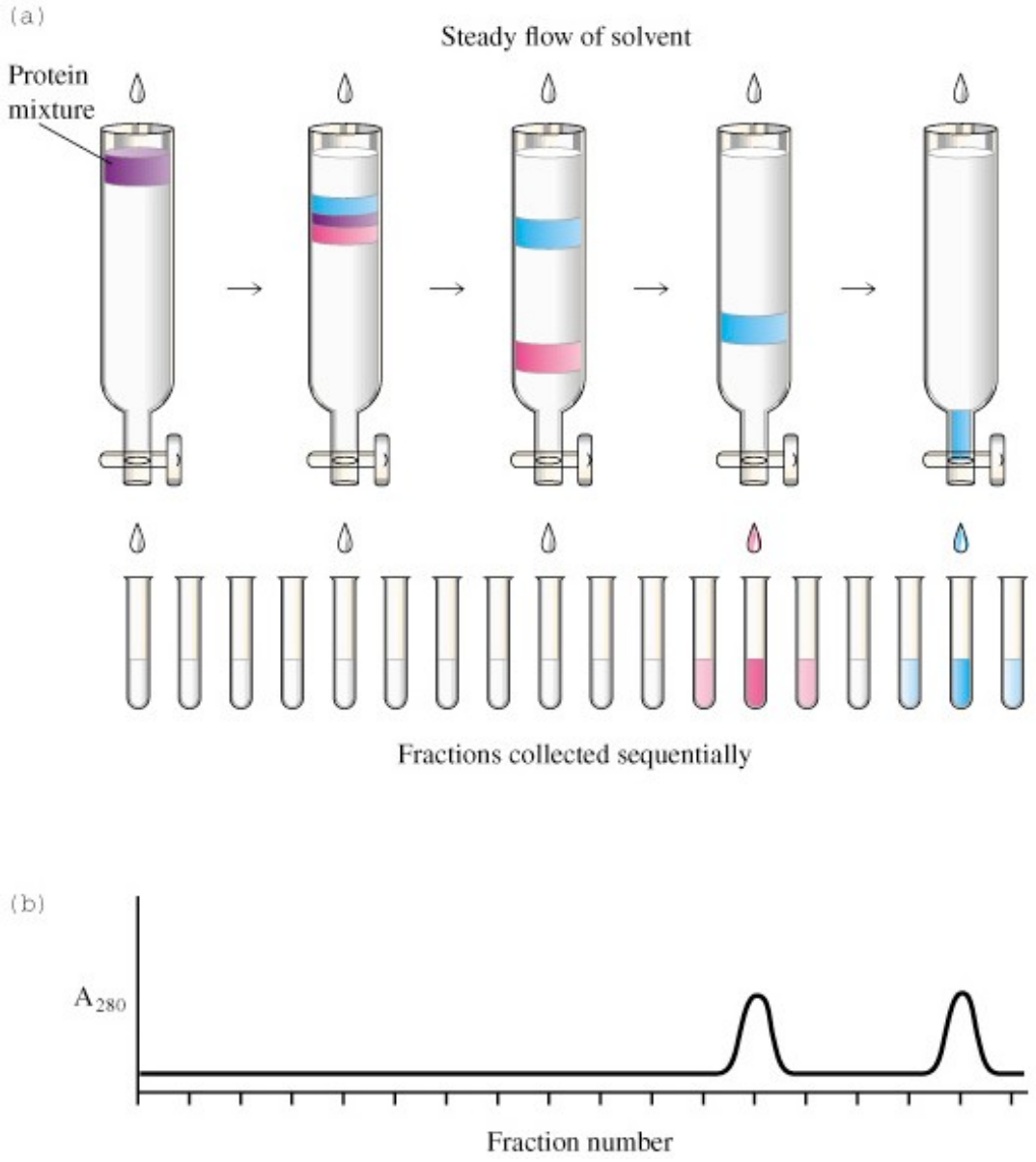
- Tipos comuns de cromatografia em coluna (HPLC):
- Cromatografia de troca iônica - separação baseada na carga geral das moléculas = eluição salina
- Cromatografia de filtração em gel - separação baseada no tamanho molecular = esferas porosas
- Cromatografia de afinidade - separação por interações de ligação específica entre a matriz da coluna e as proteínas alvo = mais seletiva

Cromatografia em coluna

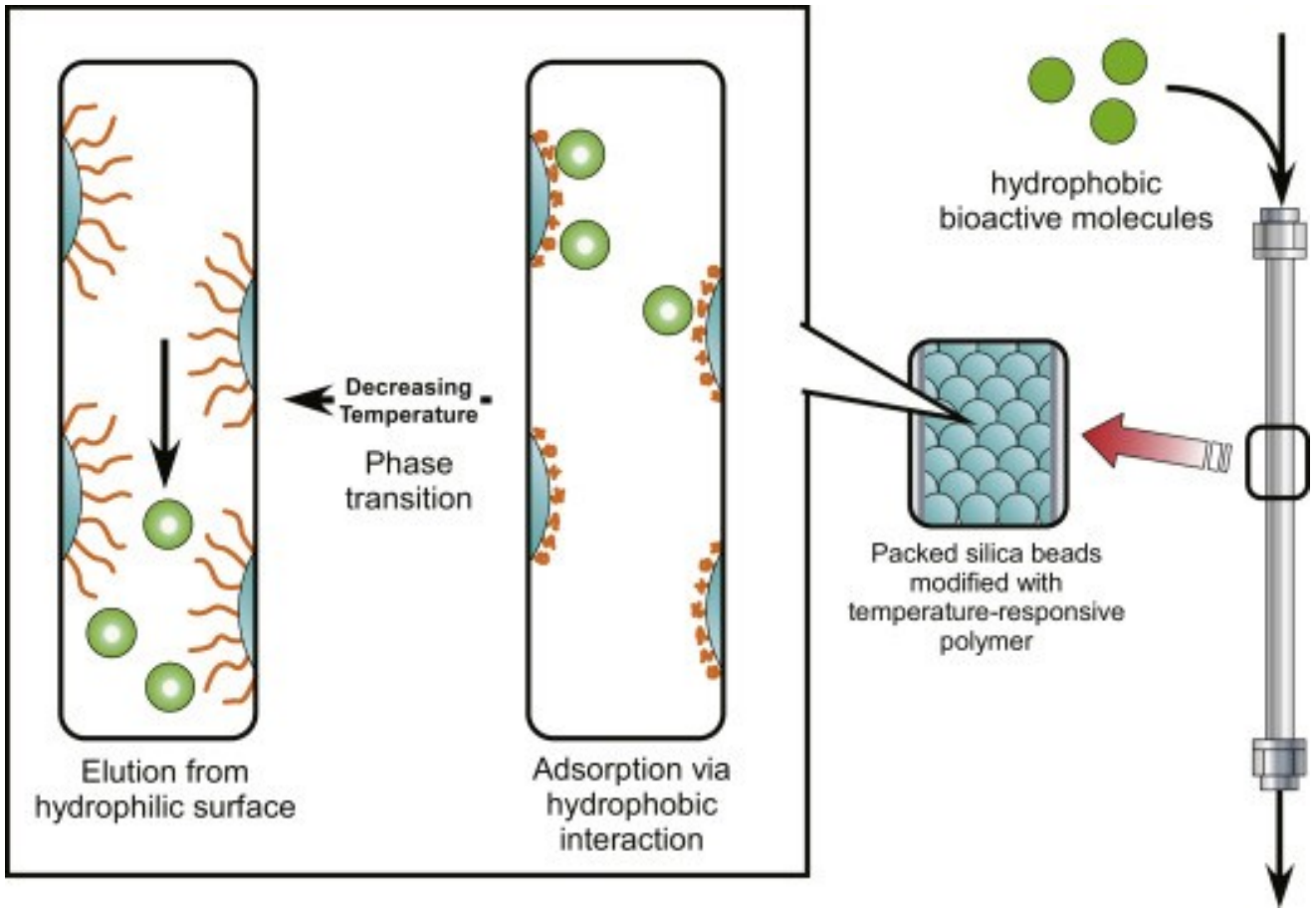
(a) Separação de uma mistura de proteínas

A concentração de proteína de cada fração é determinada medindo a absorvância a 280 nm.

(b) Detecção de picos de proteínas eluentes

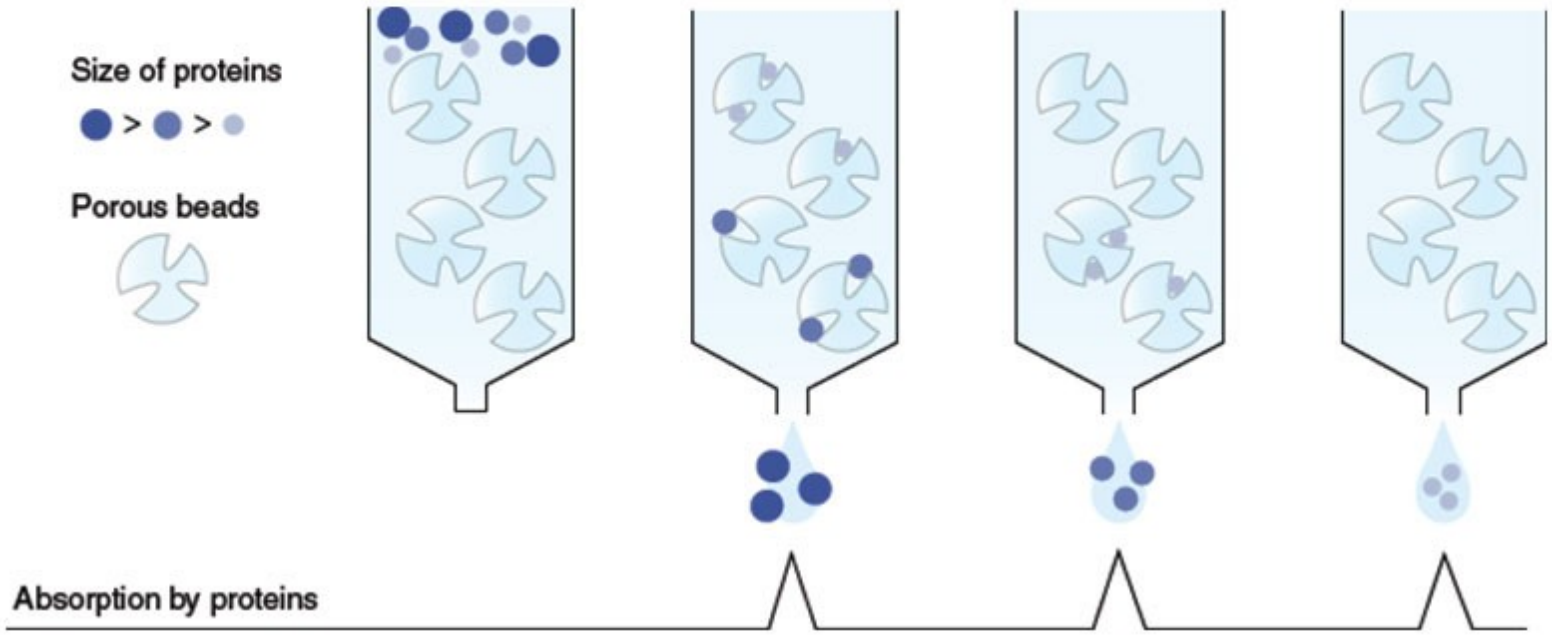


Cromatografia de fase reversa



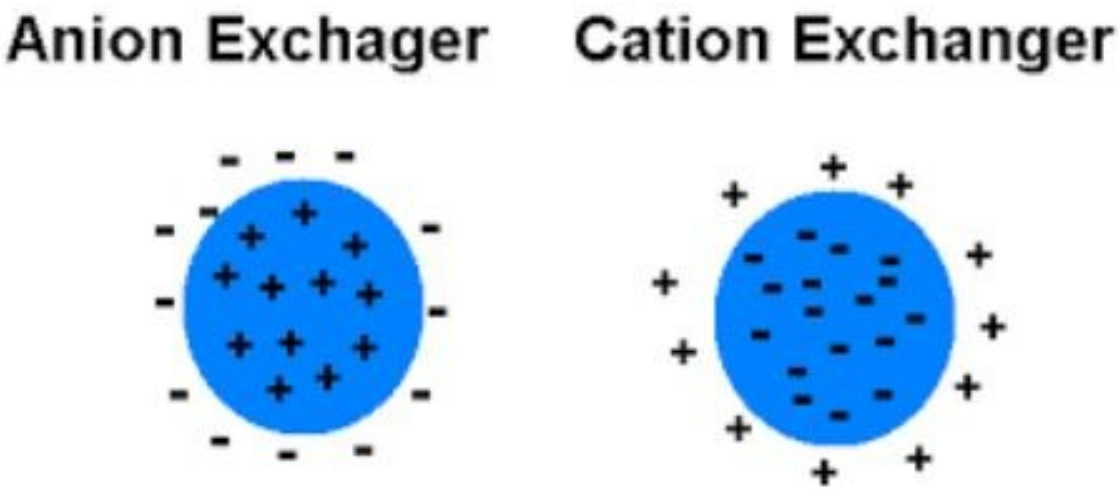
https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/reversed-phase-chromatography

Cromatografia de gel-filtração



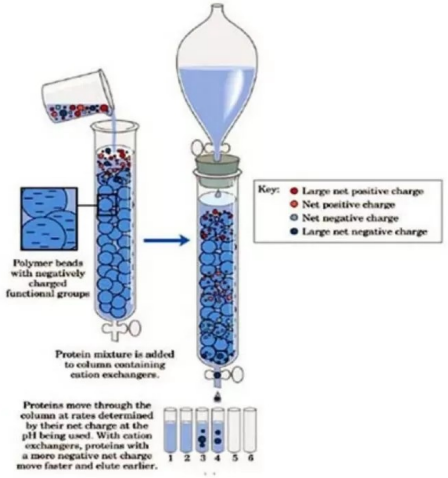
https://microbenotes.com/gel-filtration-chromatography/

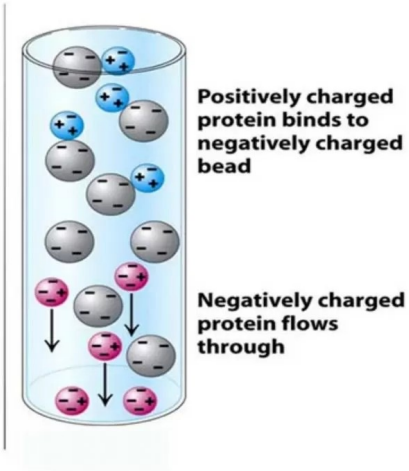
Cromatografia de troca-iônica

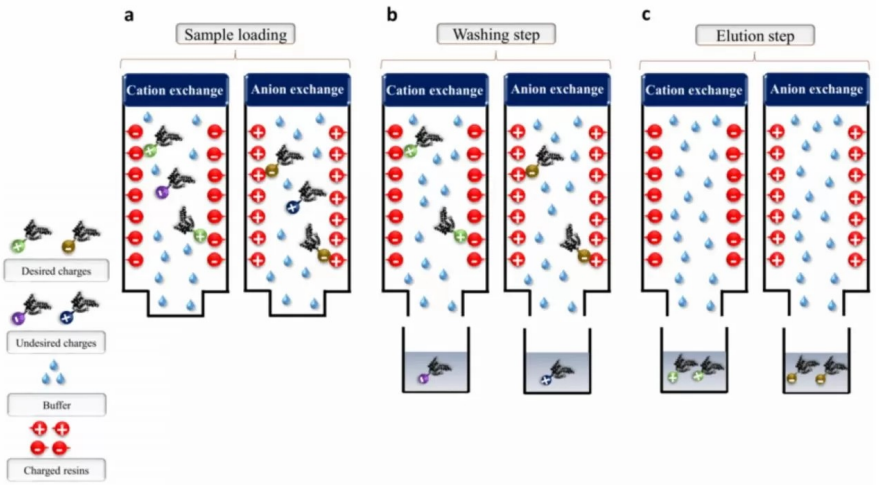


https://maestrovirtuale.com/cromatografia-de-troca-ionica-procedimento-principios/

Cromatografia de troca-iônica



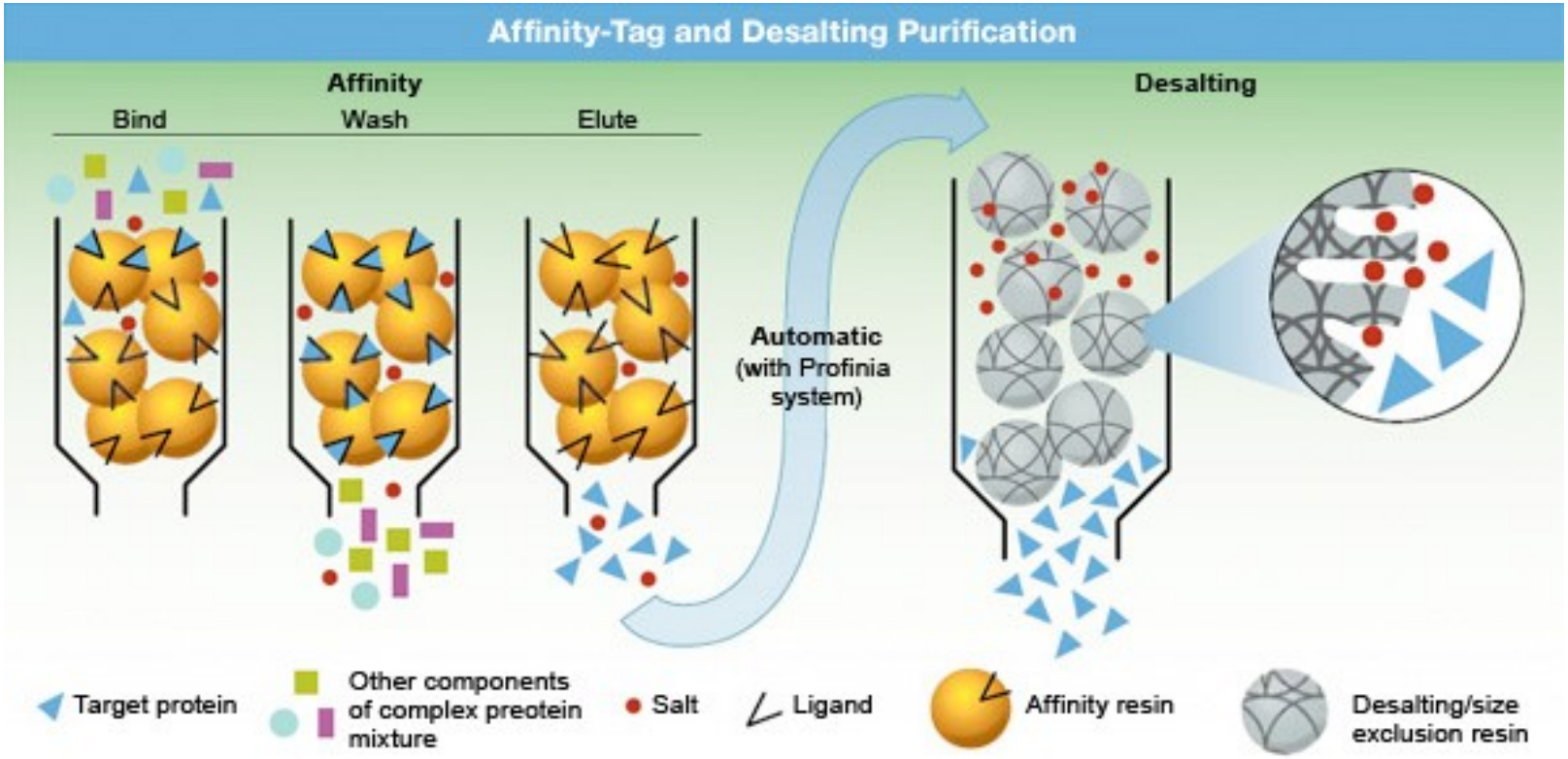




Colunas de troca-iônica

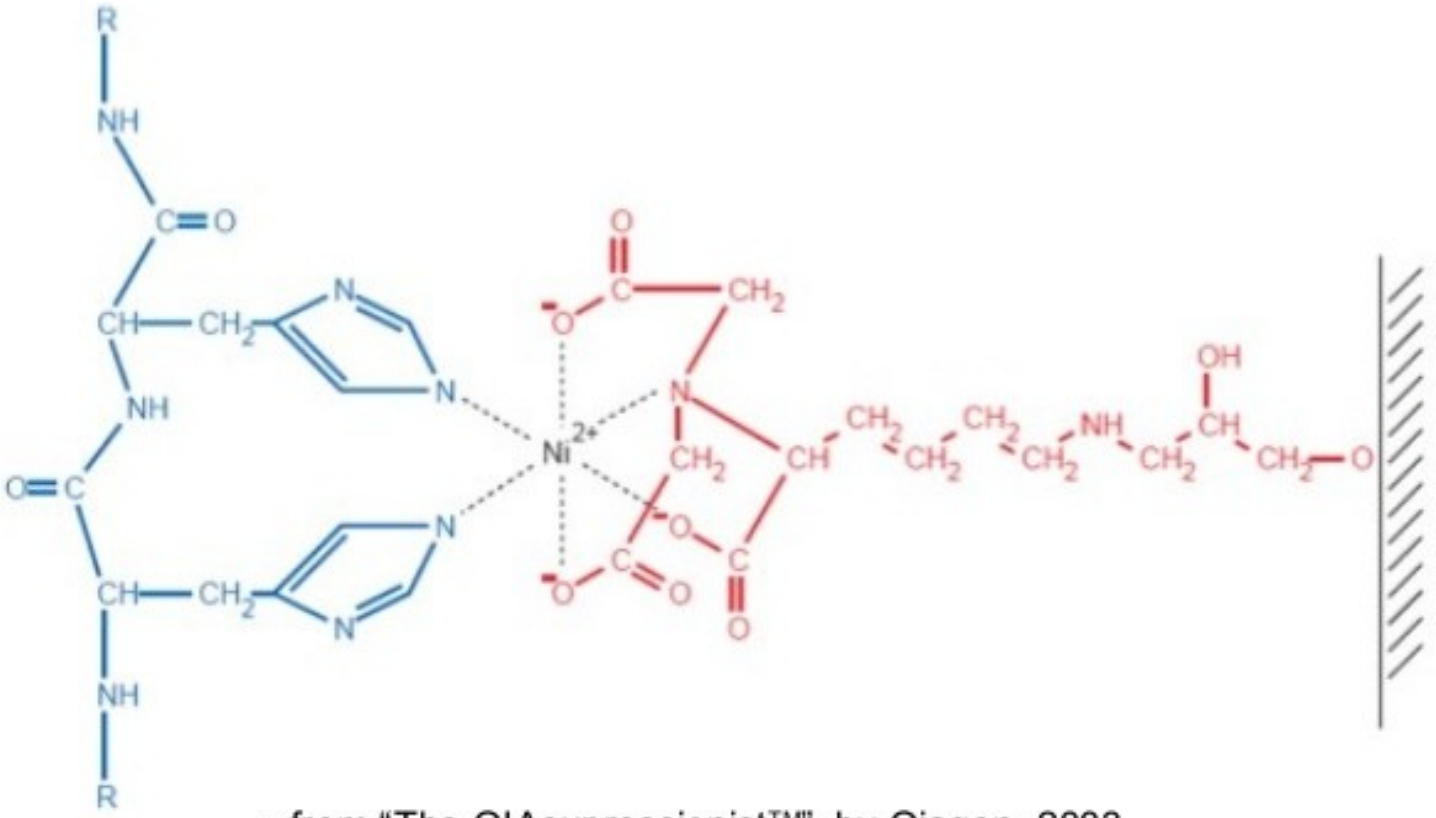
Cromatógrafo

Cromatografia de afinidade

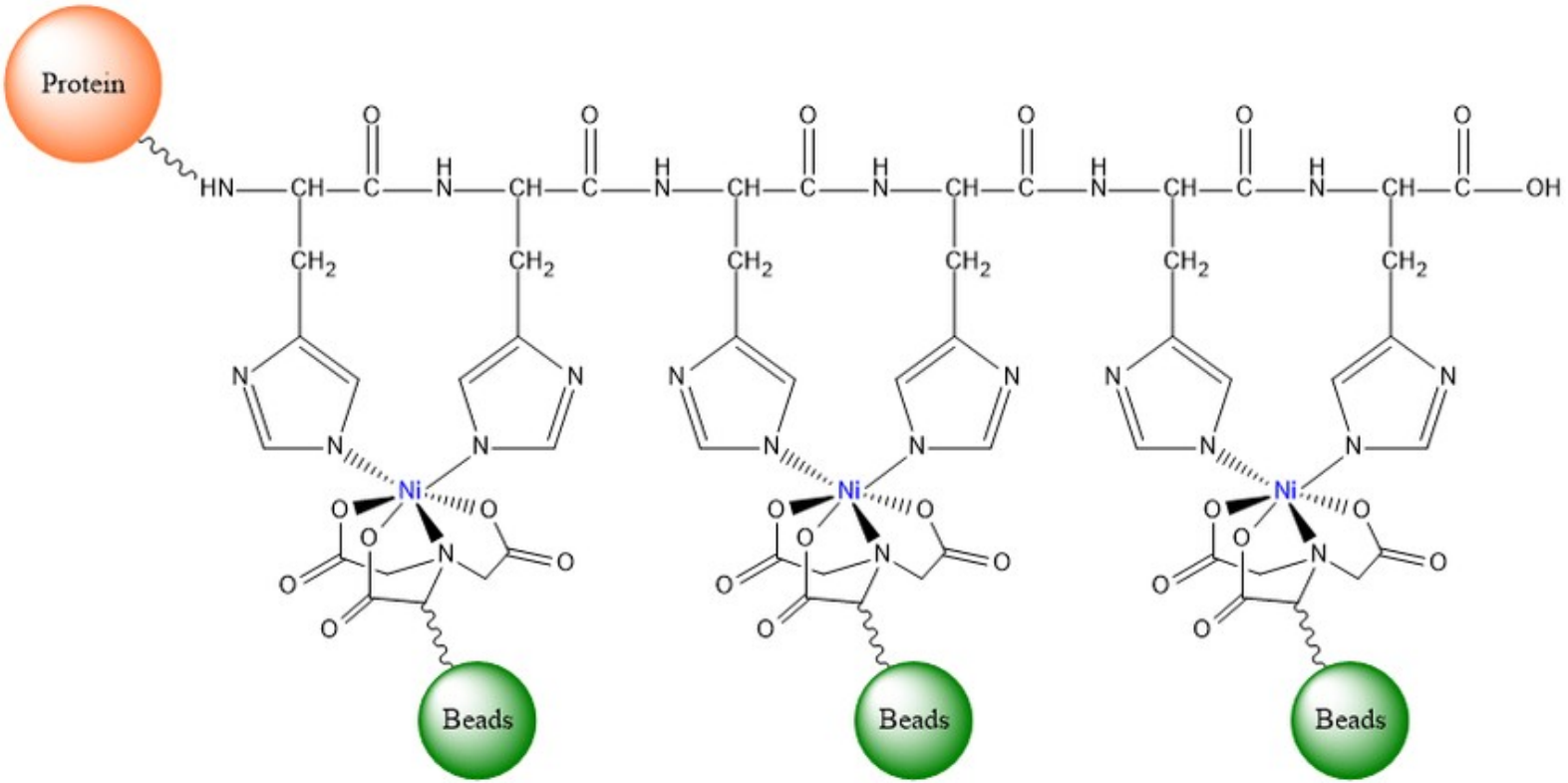


Purificação com coluna de níquel

Binding of the His-tagged protein to the Ni-NTA resin.



–from "The QIAexpressionist™", by Qiagen, 2003



Purification of Recombinant Adenoviral Hexon Proteins for Generation of Virus-specific Antibodies & Next-generation Sequencing of Adenoviral Genomes, February 2016, Thesis for: MScAdvisor: Silvio Hemmi

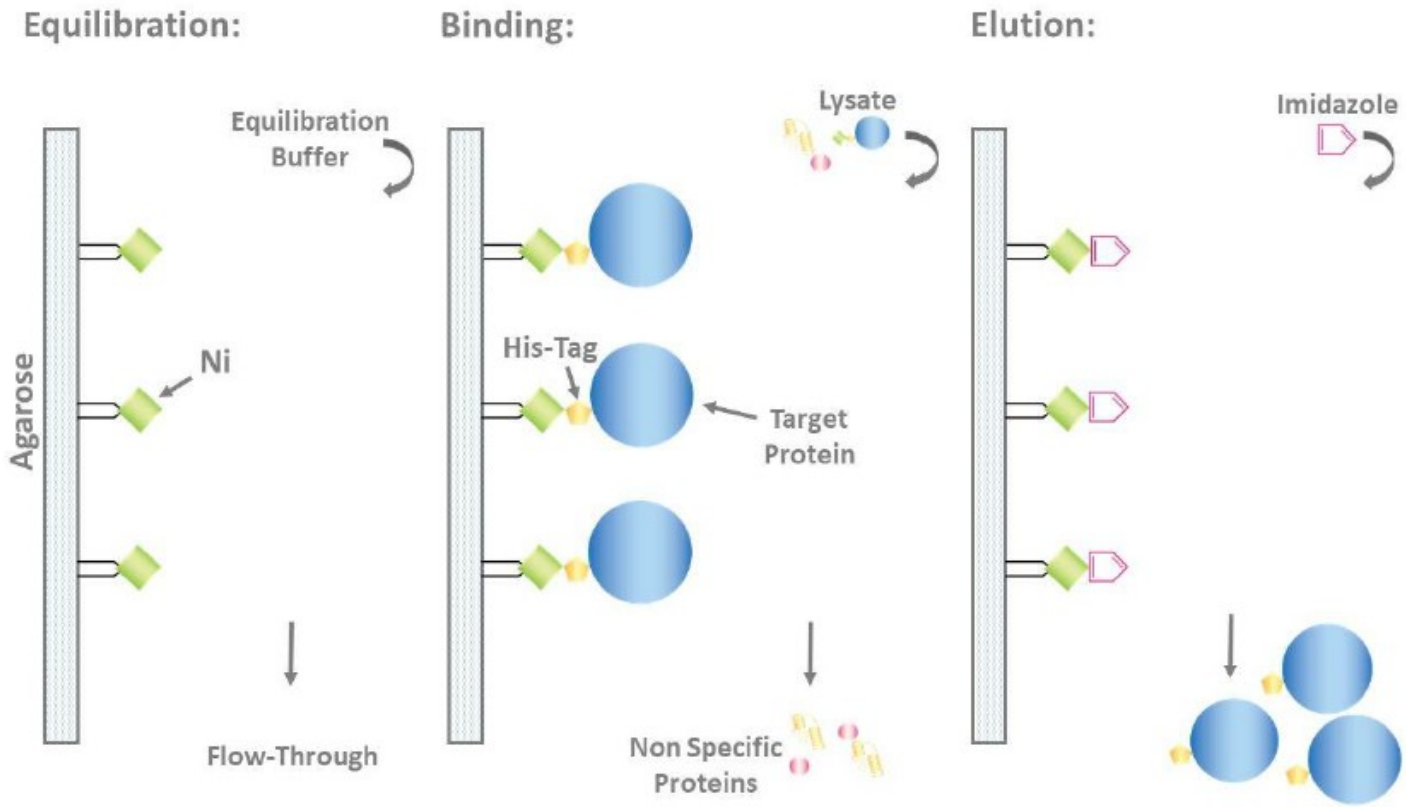


Figure 1. Purification of His-tagged target proteins using a Ni resin.

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Email: contactgoldbio86@goldbio.com

Improved Purification of Histidine-tagged Proteins with Ni Sepharose High Performance

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Purificação com coluna de níquel

Material and Methods

Chromatography conditions were, unless otherwise stated:

Medium:	Ni Sepharose High Performance prepacked in HisTrap HP 1 ml columns or packed in 5×50 mm glass columns.
Samples: Clarified	Cell extracts from *E. coli* or *Pichia. pastoris.* by centrifugation and filtration; containing 0.5 M NaCl and imidazole at a concentration appropriate for each target protein.
Flow rate:	1 ml/min.
Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, x mM imidazole, pH 7.4.
Optimization:	The optimal imidazole concentration, to obtain the best purity and yield, differs from protein to protein. A small number of screening runs, including a run with an imidazole gradient from 5 mM, will facilitate finding a suitable imidazole concentration for optimal results.
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4.

Purification of a high-MW histidine-tagged mannanase expressed in *E. coli*

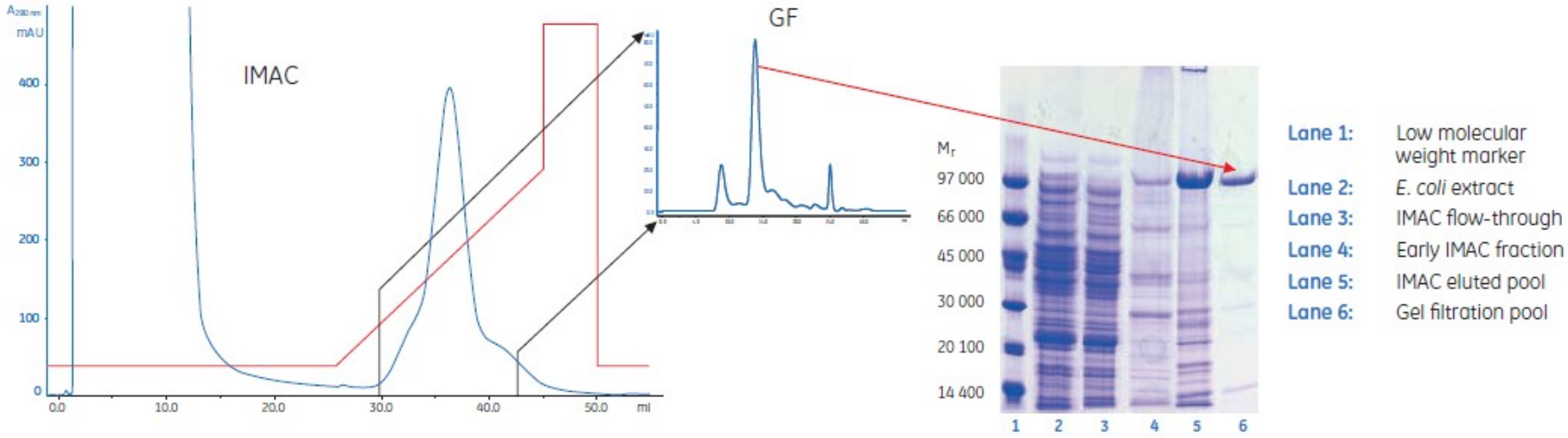


Fig 4. Purification by IMAC and gel filtration. Black lines in IMAC chromatogram indicate the IMAC pool taken to gel filtration.

- Sample: *E. coli* extract with low-level expression of a histidine-tagged mannanase, Man 26A, from *Cellulomonas fimi* (M_r ~ 100 000).
- IMAC: 10 ml extract applied to a 1 ml column with Ni Sepharose High Performance. 30 mM imidazole during equilibration, sample application, and wash (see Optimization in Materials and Methods). Elution with a 25 ml linear imidazole gradient to 300 mM.
- Gel filtration: Superdex™ 200 10/300 GL.

Results

- Enzymatically active purified Man 26A.

Note: Several contaminants were co-purified with the full-length target protein. Previous results have indicated that these contaminants include various truncated, histidine-tagged forms of the target protein (data not shown). Accordingly, a second purification step was performed.

Purification of a histidine-tagged hydrolase expressed in *Pichia pastoris*

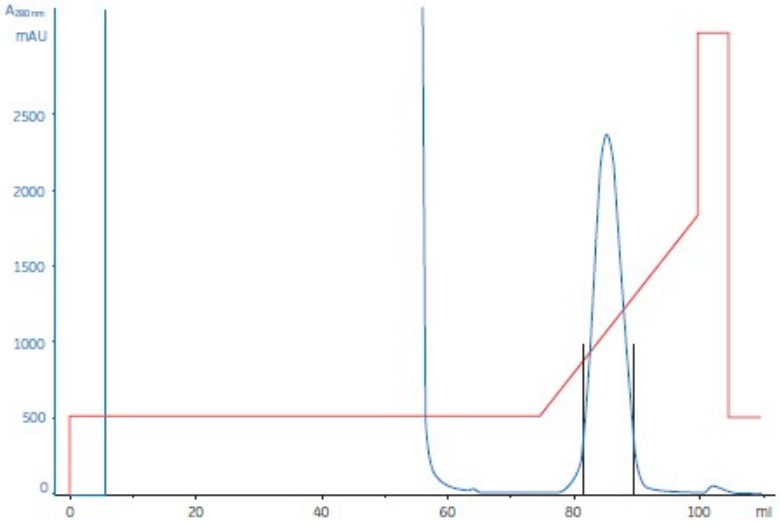
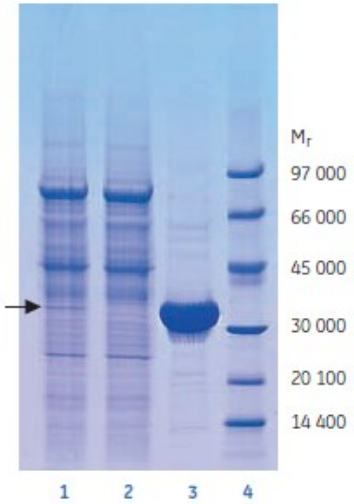


Fig 5. Black lines in chromatogram indicate the final pool. Arrow indicates the target protein band in the *Pichia* extract.



Lane 1: *Pichia* extract
Lane 2: Flow-through
Lane 3: Final pool
Lane 4: Low molecular weight marker

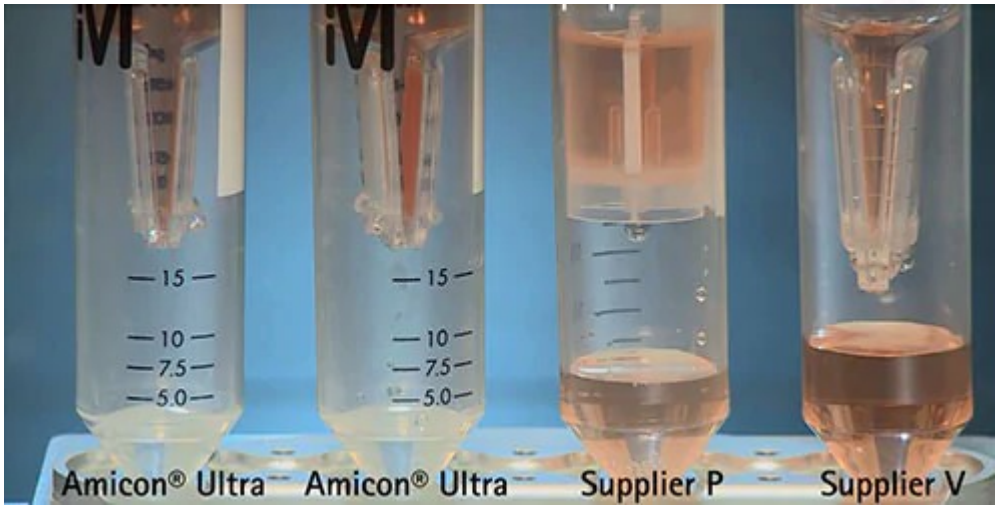
IMAC: 50 ml cell extract (corresponding to 20 g cells) applied to a 1 ml column with Ni Sepharose High Performance. 85 mM imidazole during equilibration, sample application and wash (see Optimization in Materials and Methods). Elution with a 25 ml linear imidazole gradient to 300 mM.

Fig 6. SDS-PAGE analysis of *Pichia pastoris* extract with low-level expression of a (putative) hydrolase from *Saccharomyces cerevisiae* (M_r ~ 34 000).

Results

- High-purity target protein was obtained from a low-expression sample.
- The Ni^{2+} concentration was low in the final pool of purified protein, 155 ppb (molar Ni^{2+}/protein ratio = 0.08).

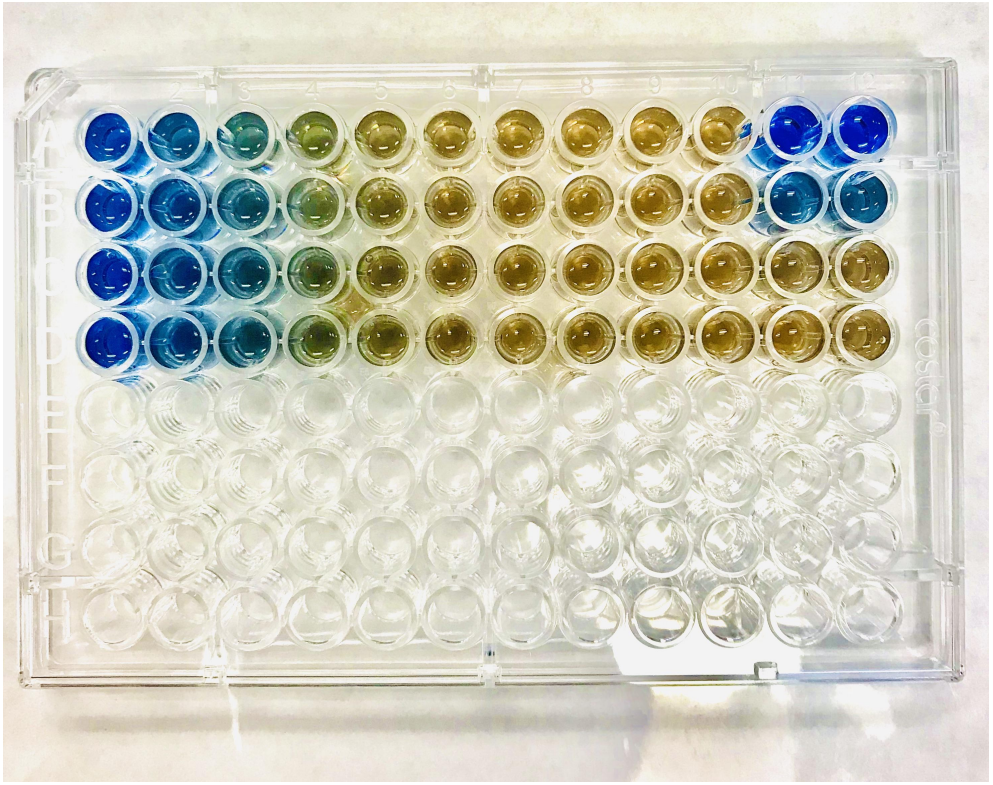
Diálise/ concentração



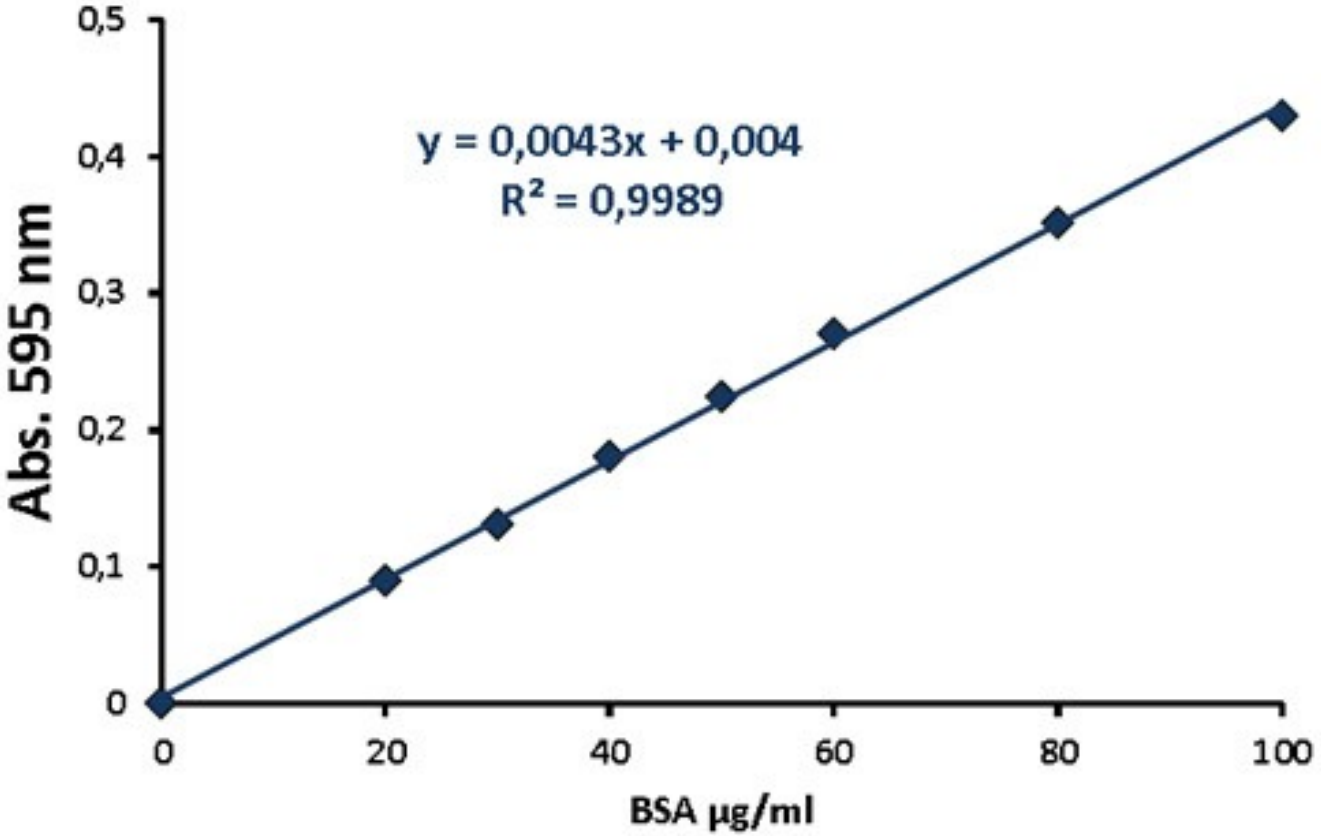
Ensaio de Bradford

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Ensaio de Bradford



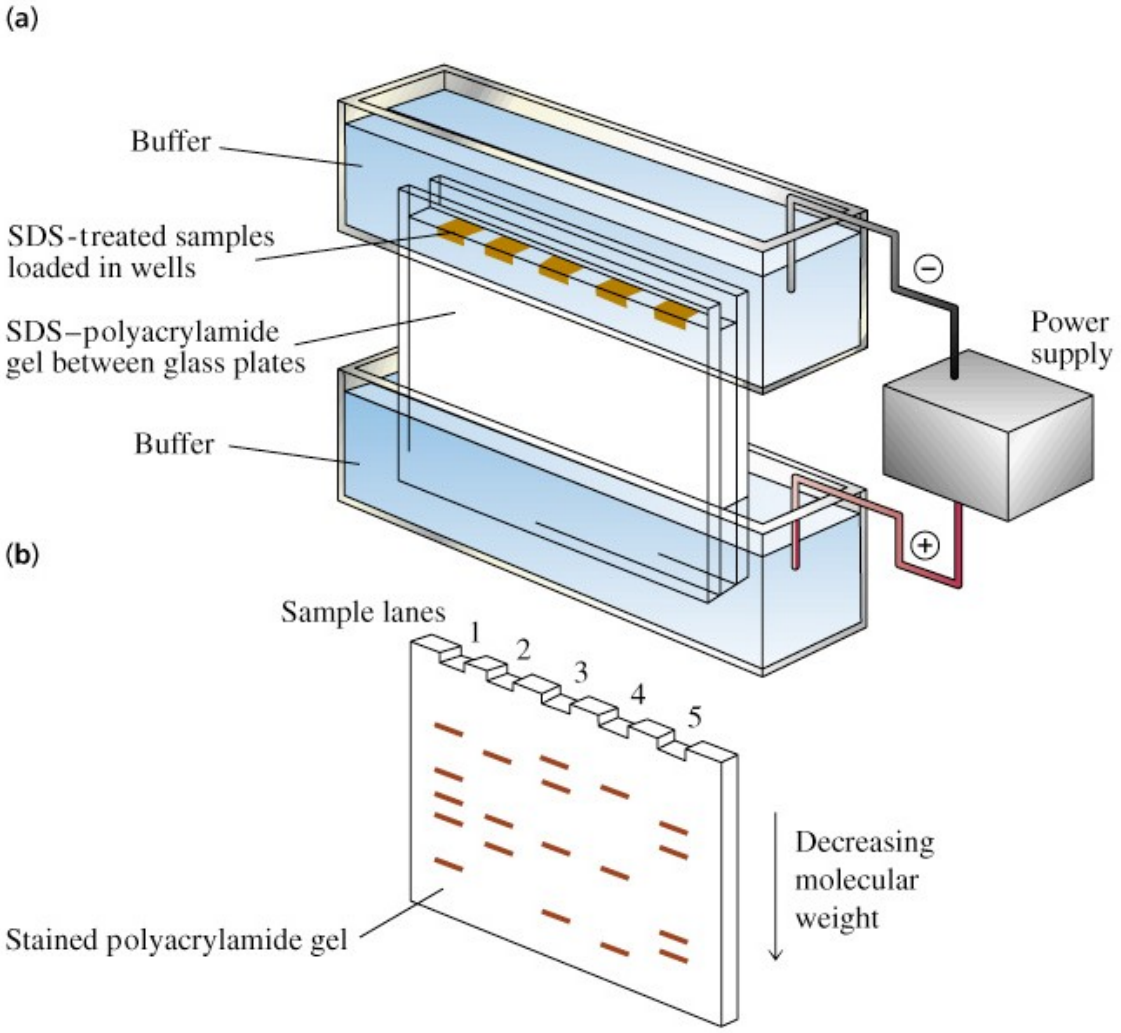
Ensaio de Bradford

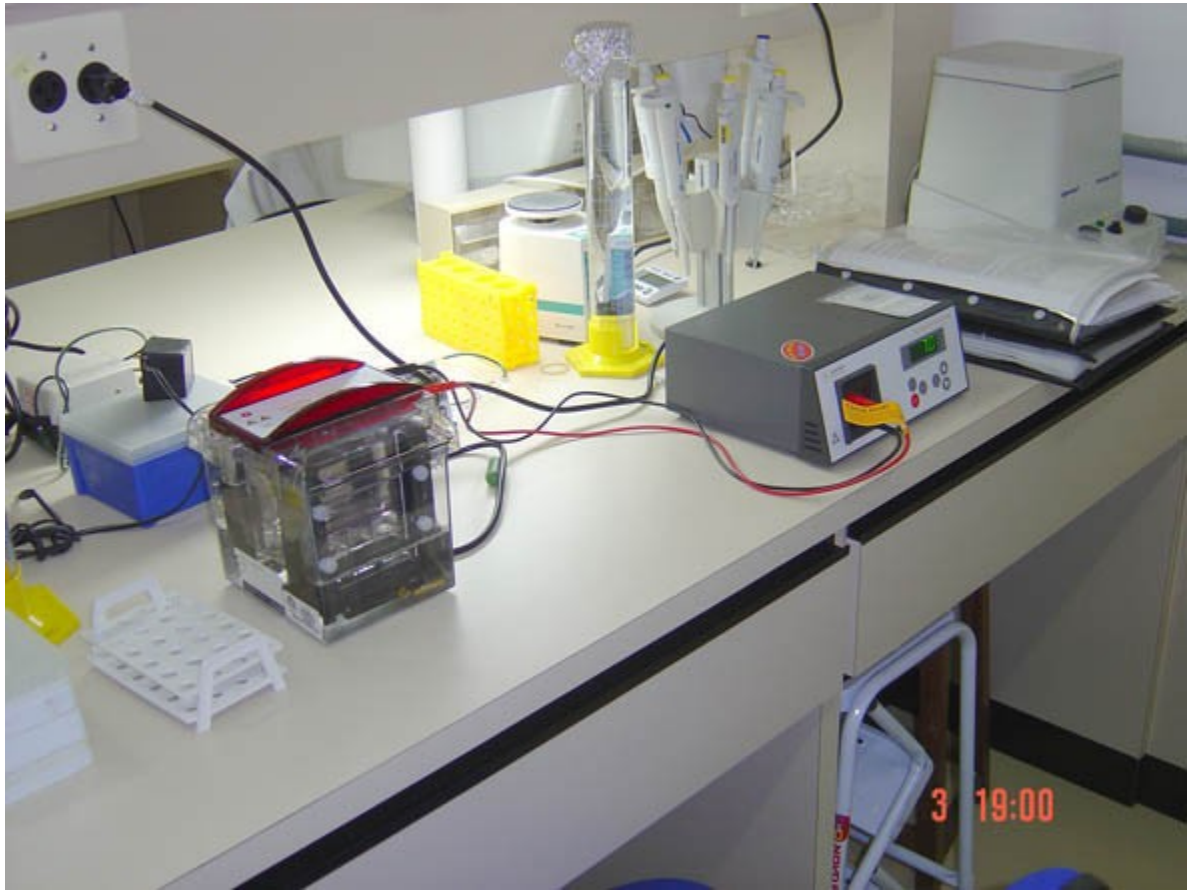


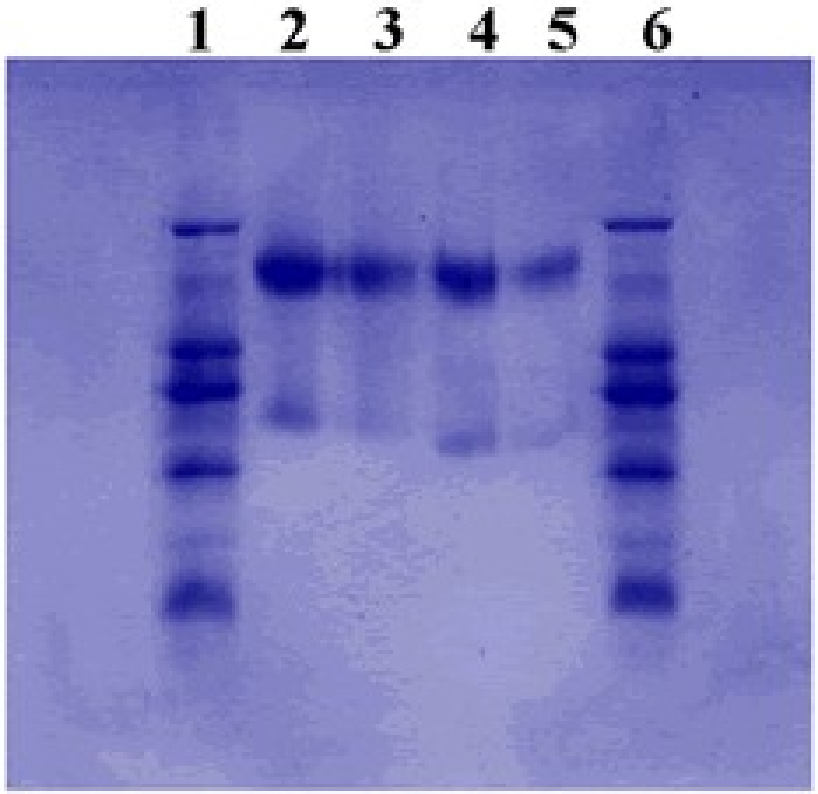
Eletroforese

- Eletroforese em gel de poliacrilamida (PAGE) Separa moléculas carregadas por tamanho (peso molecular) em uma matriz de gel de poliacrilamida quando um campo elétrico é aplicado.
- SDS. O dodecilsulfato de sódio (SDS) reveste proteínas com cargas negativas. Cadeias polipeptídicas revestidas são então separadas por massa molecular (método para determinar o peso molecular)

(a) SDS-PAGE
(b) Padrão de bandas de proteínas após corrida



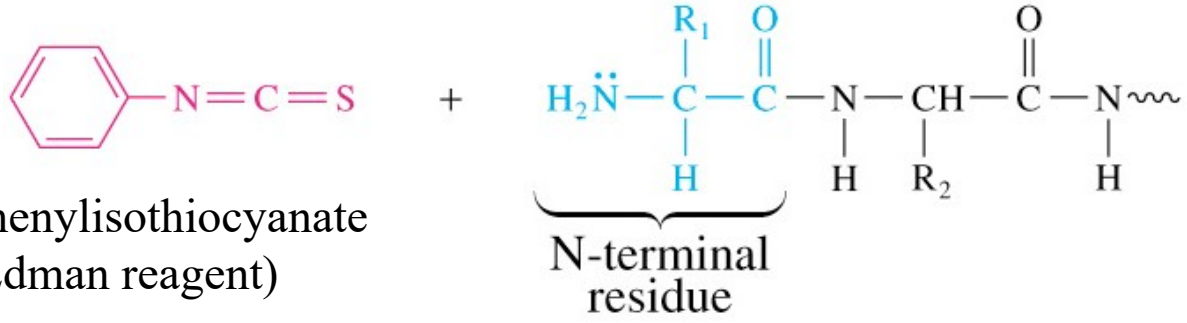

3 19:00


1
2
3
4
5
6

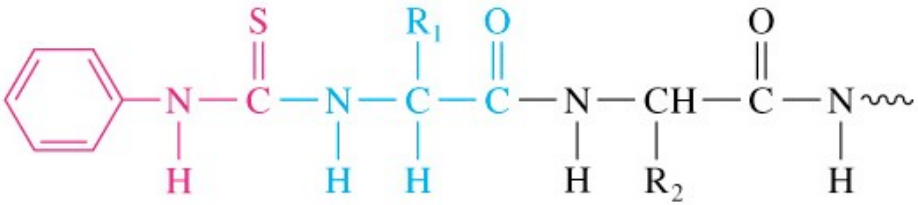
Determinando a sequência de aminoácidos

- Procedimento de degradação de Edman - Determinação de um resíduo de cada vez a partir do terminal N
- (1) Tratar o peptídeo com PITC que reage com o terminal N para formar um peptídeo PTC
- (2) Trate com ácido trifluoroacético (TFA) para
- clivar seletivamente a ligação peptídica N-terminal
- (3) Separar o derivado N-terminal do peptídeo
- (4) Converter derivado em aminoácido PTH

Procedimento de degradação de Edman



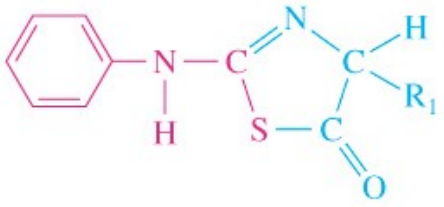
pH = 9.0



Phenylthiocarbamoyl-peptide

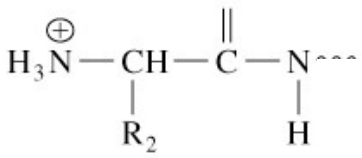
F_3CCOOH

Procedimento de degradação de Edman



Anilinothiazoli

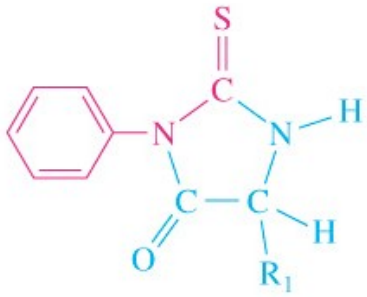
+



Polypeptide chain with

Aqueous acid

Aqueous



Phenylthiohydantoin derivative

Amino acid identified

Returned to alkaline conditions for reaction with additional

Polypeptide chain with n-1 amino acids

Returned to alkaline conditions for reaction with additional phenylisothiocyanate in the next cycle of Edman degradation

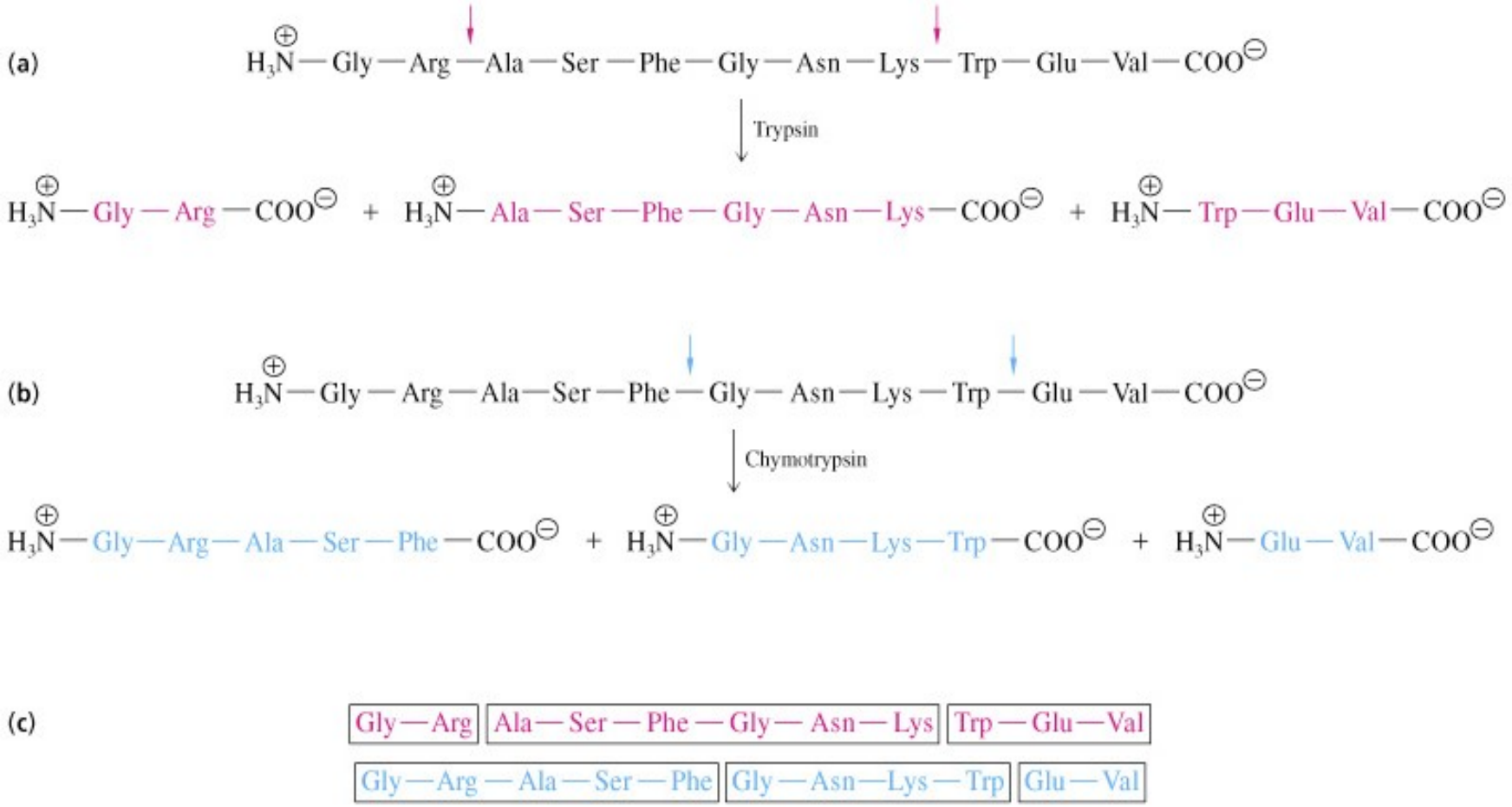
Estratégias de sequenciamento de proteínas

- As proteínas podem ser muito grandes para serem sequenciadas completamente pelo método de Edman
- Proteases (enzimas que clivam ligações peptídicas) e agentes químicos são usados para clivar seletivamente a proteína em fragmentos menores.
- O brometo de cianogênio (BrCN) cliva polipeptídeos no terminal C dos resíduos Met

Enzimas protease clivam ligações peptídicas específicas em outros locais do peptídeo

- Quimotripsina - lado carbonílico de resíduos alifáticos não carregados aromáticos ou volumosos (por exemplo, Phe, Tyr, Trp, Leu)
- Tripsina - lado carbonílico, resíduos básicos (Lys,Arg).
- Protease de Staphylococcus aureus V8 - lado carbonila de resíduos carregados negativamente (Glu, Asp). NOTA: em bicarbonato de amônio 50mM cliva apenas em Glu.

Clivagem, sequenciamento de um oligopeptídeo
A sequência de aminoácidos de uma grande cadeia polipeptídica pode ser determinada alinhando sequências correspondentes de fragmentos peptídicos sobrepostos



Sequenciamento por espectrometria de massa

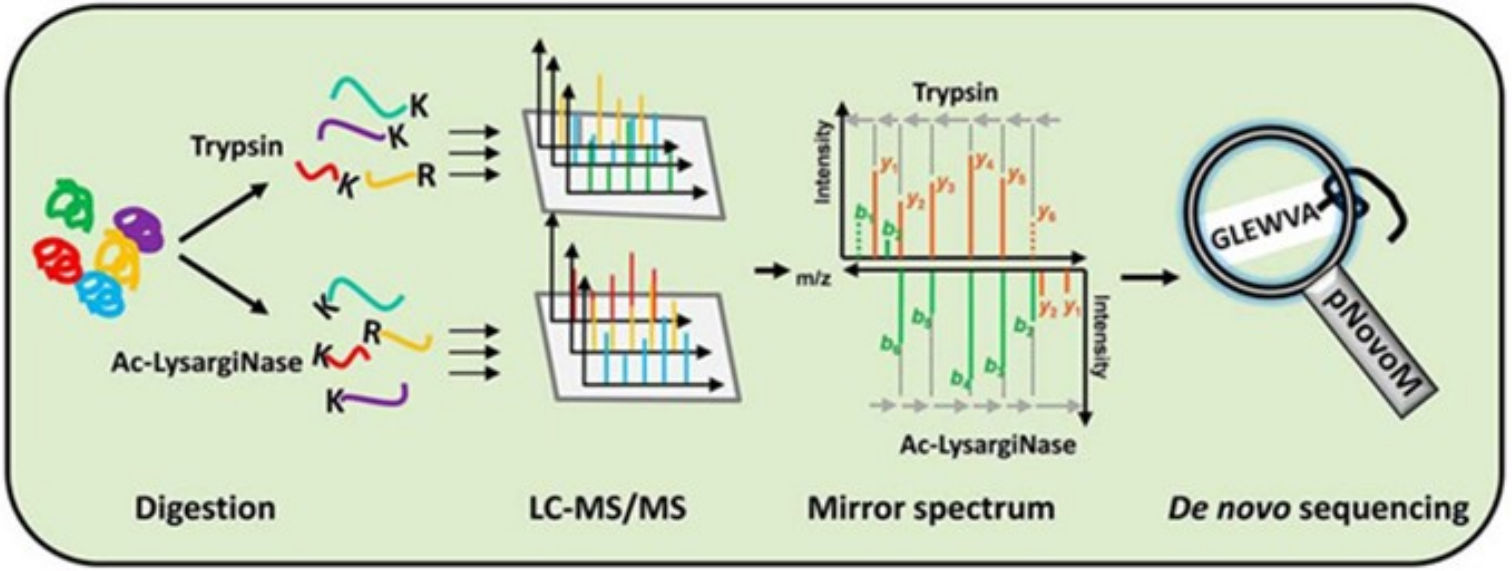


Figure 1. The process of peptide de novo sequencing by mass spectrometry (Hao, et al. 2019).

Sequenciamento por espectrometria de massa