

Metodologija rada sa proteinima

- Izolovanje i prečišćavanje proteina
- Karakterisanje proteina
- Ispitivanje i (evt.) primena izolovanog proteina

Izolovanje i prečišćavanje proteina

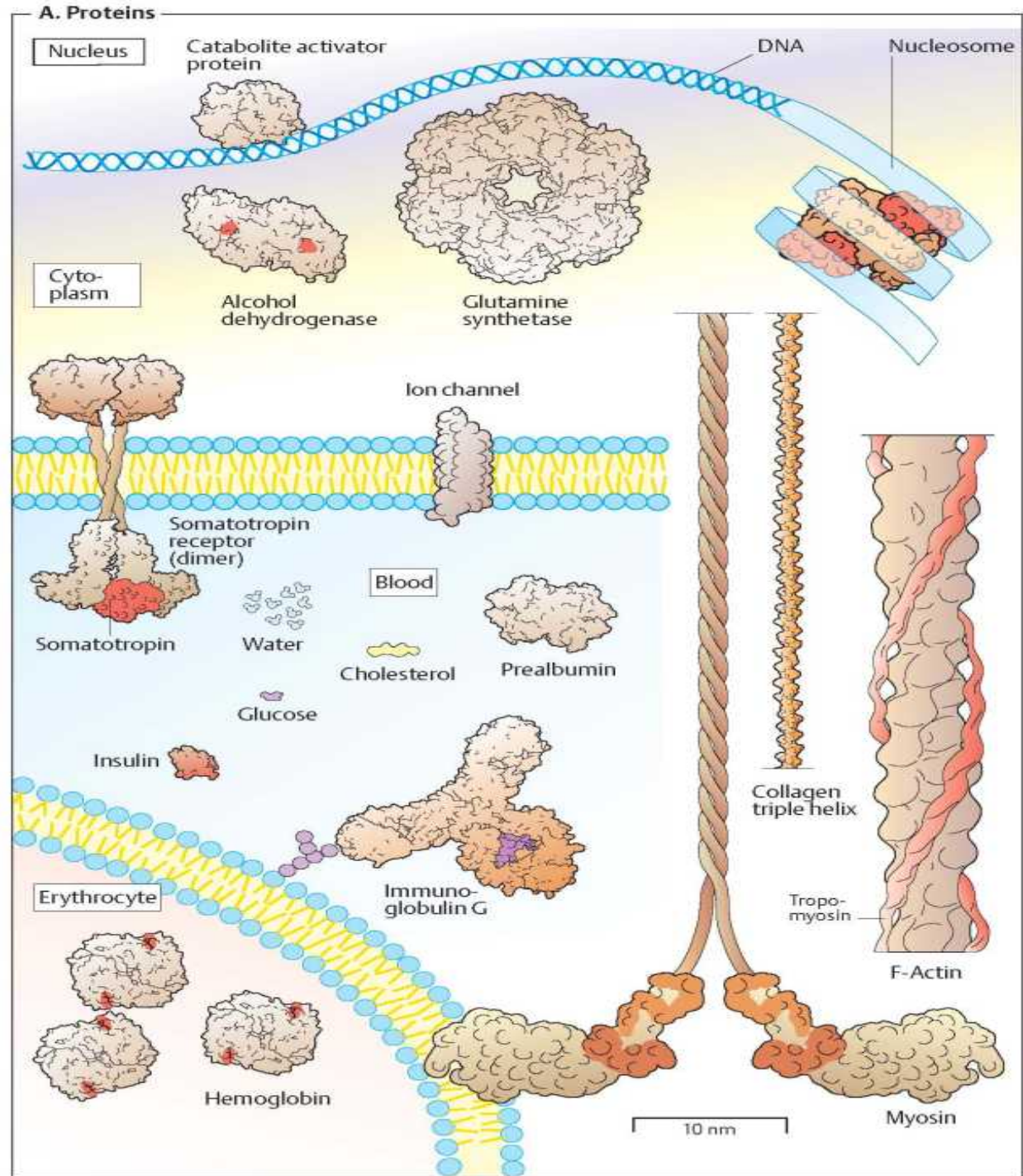
“Never waste pure thoughts on an impure protein”

- Najčešće korišćeni polazni materijal
- Kako prepoznati protein koji tražimo?
- Dezintegracija ćelija i ekstrakcija proteina
- Frakcionisanje sirovog ekstrakta
- Određivanje koncentracije proteina (vežbe BPNK)
- Određivanje (praćenje) čistoće (homogenosti)
- Određivanje aktivnosti (kursevi na višim godinama)
- Kristalizacija (BPNK: predavanje 4/11)

Najčešće korišćeni polazni materijal:

- pacov (proteini jetre)
- velike životinje (proteini srca, timusa, mozga)
- zec (mišićni proteini)
- humana krv (proteini plazme, krvnih ćelija)
- mikroorganizmi (E.coli, kvasac):
tehnologija rekombinantne DNK!!!

Kako prepoznati
protein koji
tražimo?



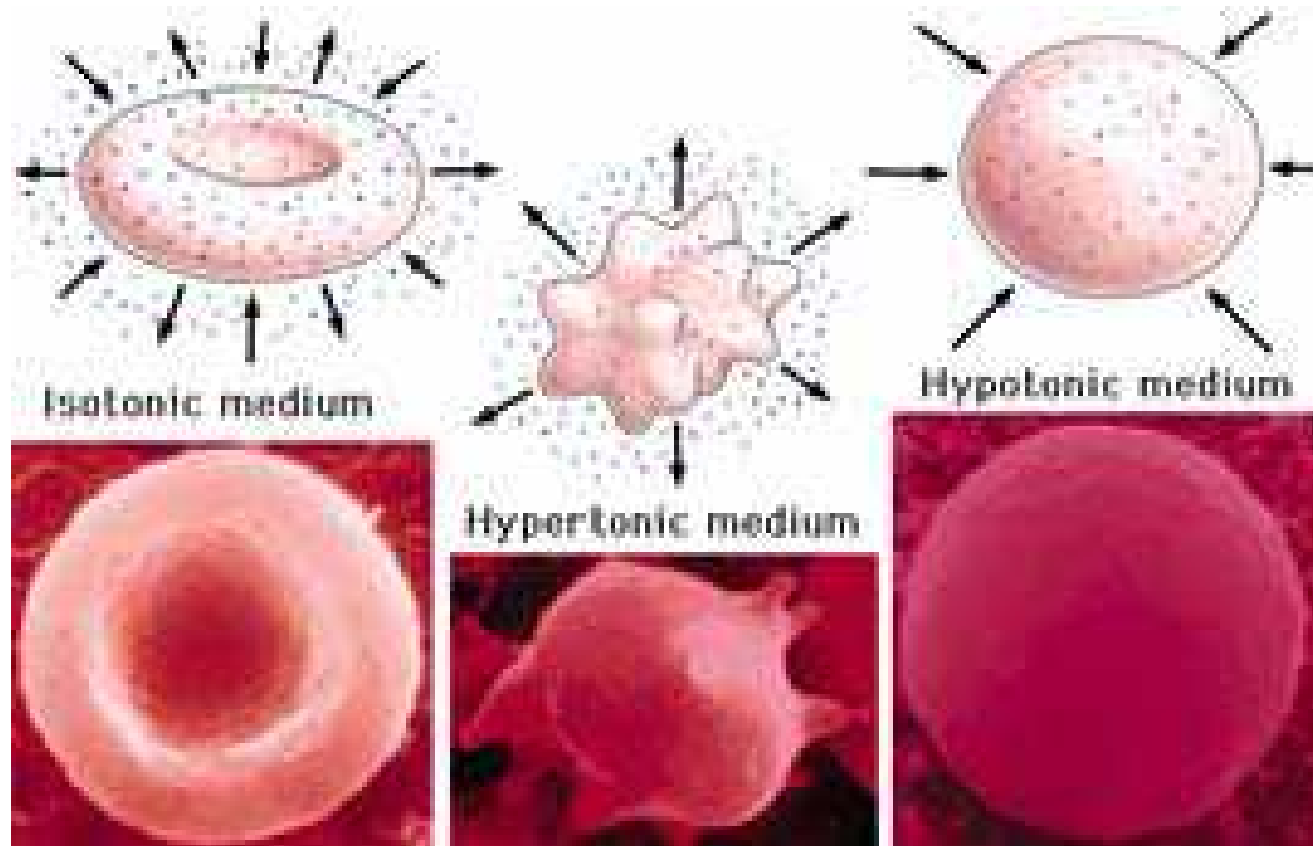
Kako prepoznati protein koji tražimo?

- Protein koji izolujemo obično predstavlja mali deo (na primer 1%!) od polaznog materijala!
- Kako znamo gde se nalazi “naš” protein?
- Potreban je specifični test (“assay” = proba) za određenu jedinstvenu osobinu (na primer aktivnost) datog proteina: primer enzimi!!!!
- Kako znamo koliko ima datog proteina u uzorku?
 - potrebno je odrediti koncentraciju proteina i
 - odrediti specifičnu aktivnost (aktivnost/mg proteina)

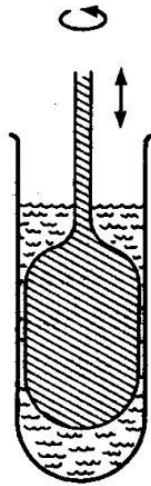
Dezintegracija ćelija i ekstrakcija proteina

- Dezintegracija ćelija
- Izolovanje organela
- Solubilizacija membranskih proteina pomoću detergenata

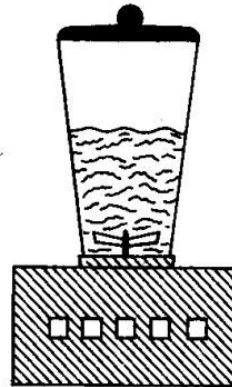
Dezintegracija ćelija – osmotska liza



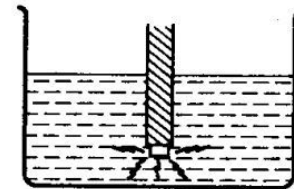
Uređaji za dezintegraciju ćelija



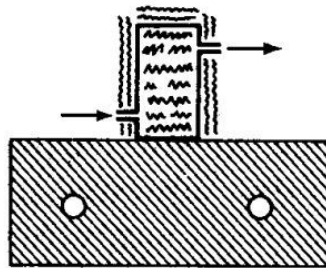
(a) Hand-operated
or motor-driven



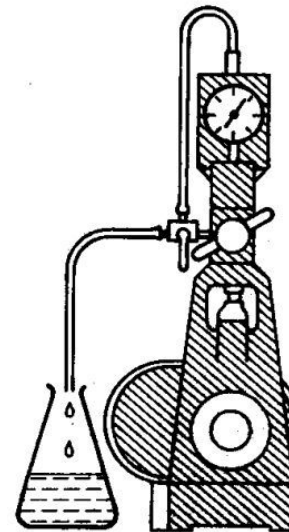
(b) Waring blender



(c) Ultrasound



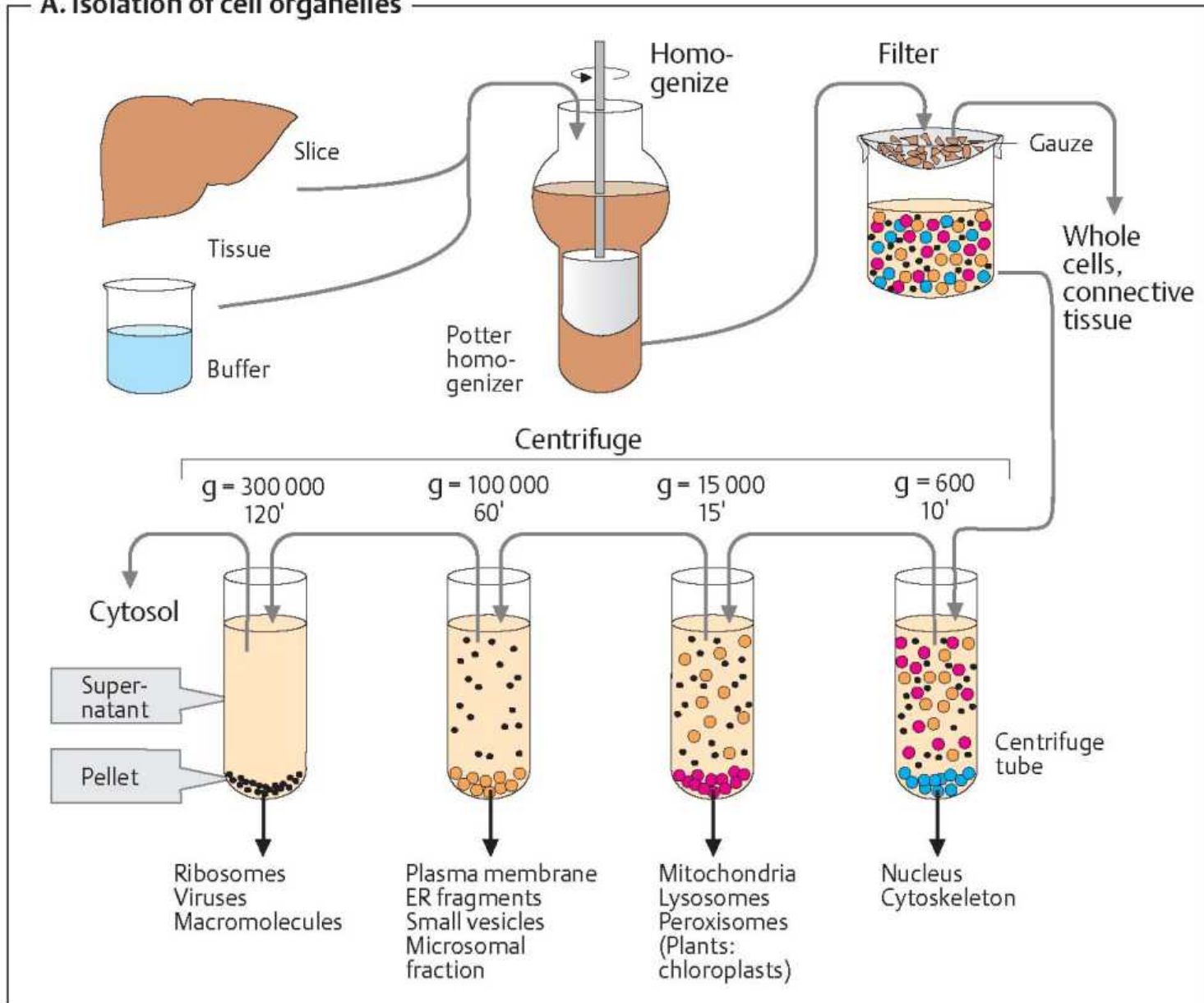
(d) Vibrating bead mill



(e) Manton-Gaulin homogenizer

Izolovanje organela

A. Isolation of cell organelles

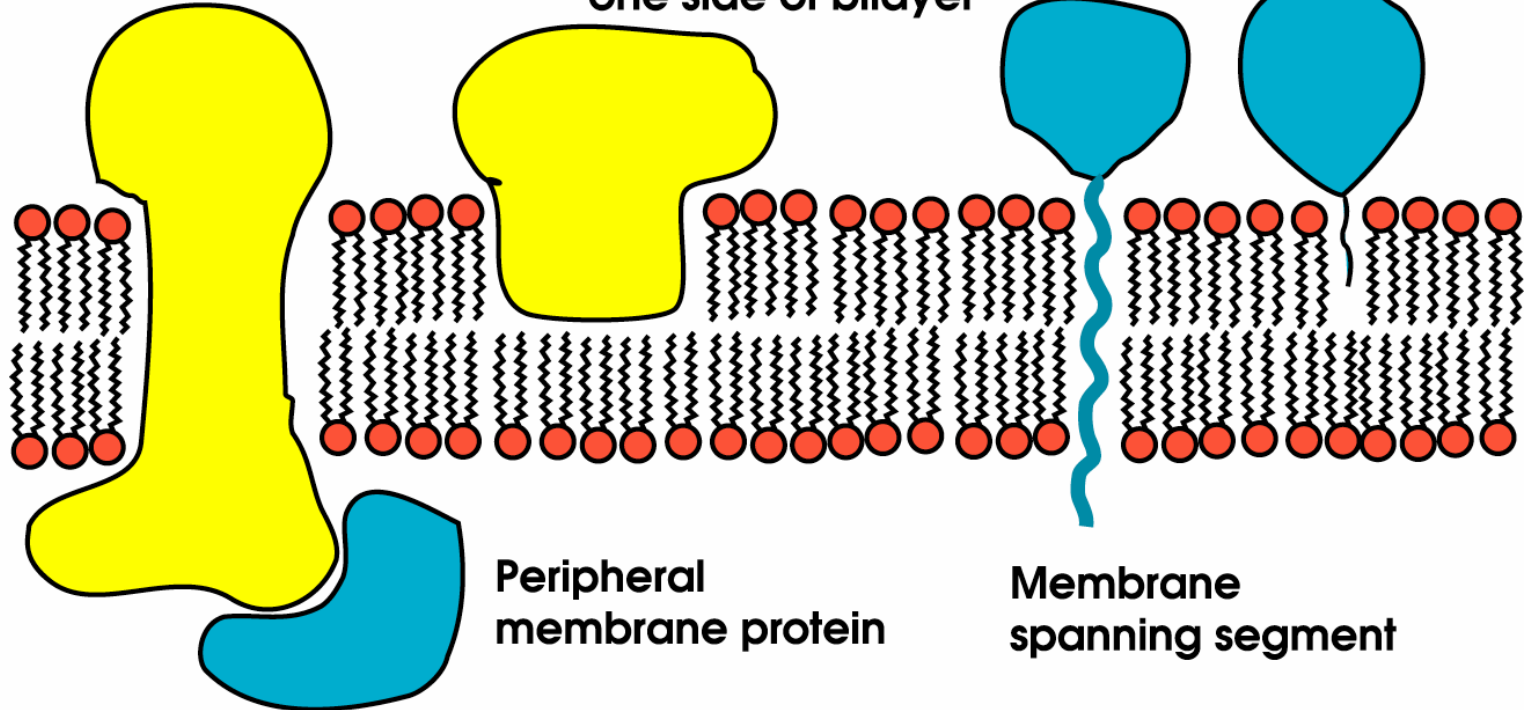


Solubilizacija membranskih proteina pomoću detergenata

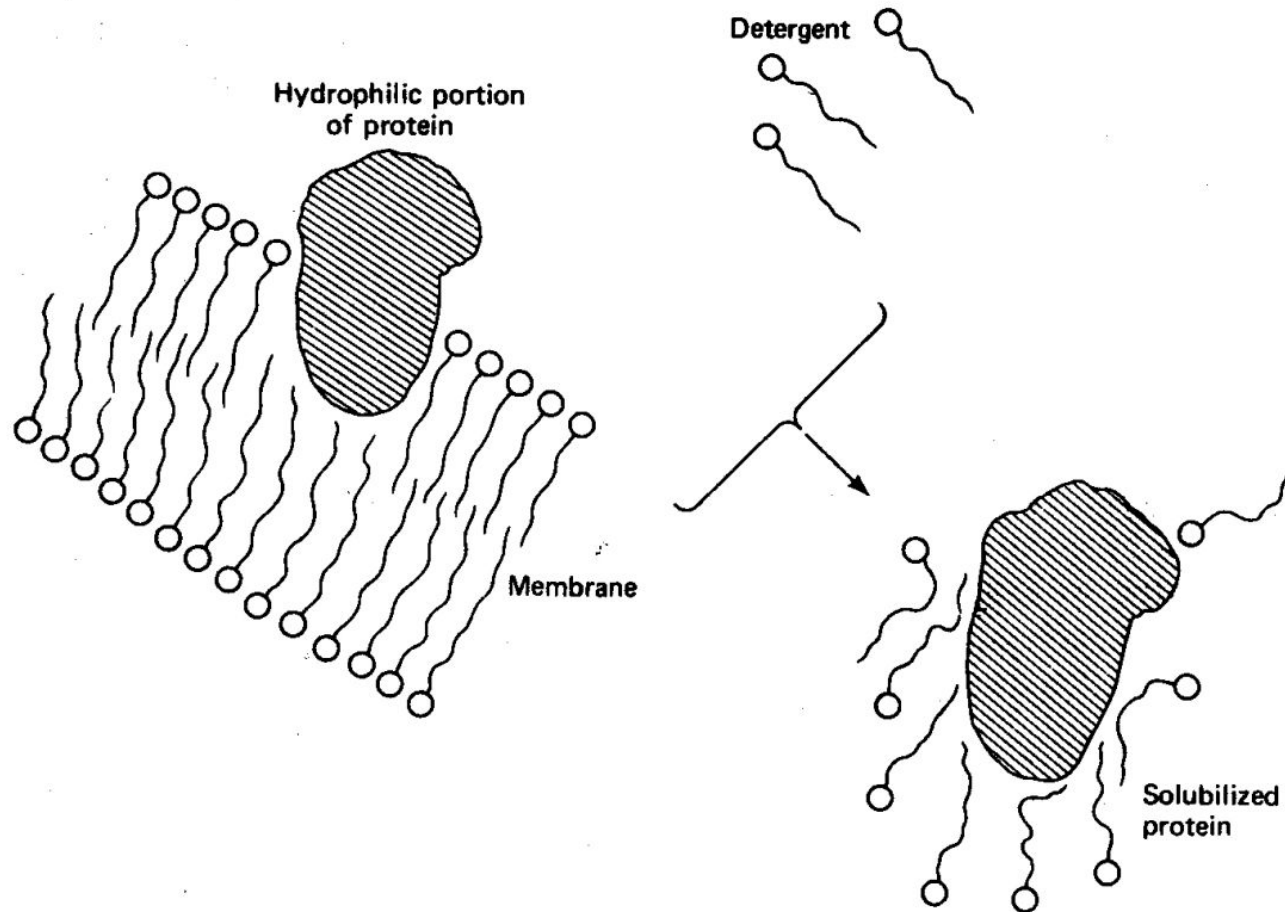
Protein Spans the bilayer and has domains on both sides

Protein is inserted in one side of bilayer

Membrane Anchor



Solubilizacija membranskih proteina pomoću detergenata

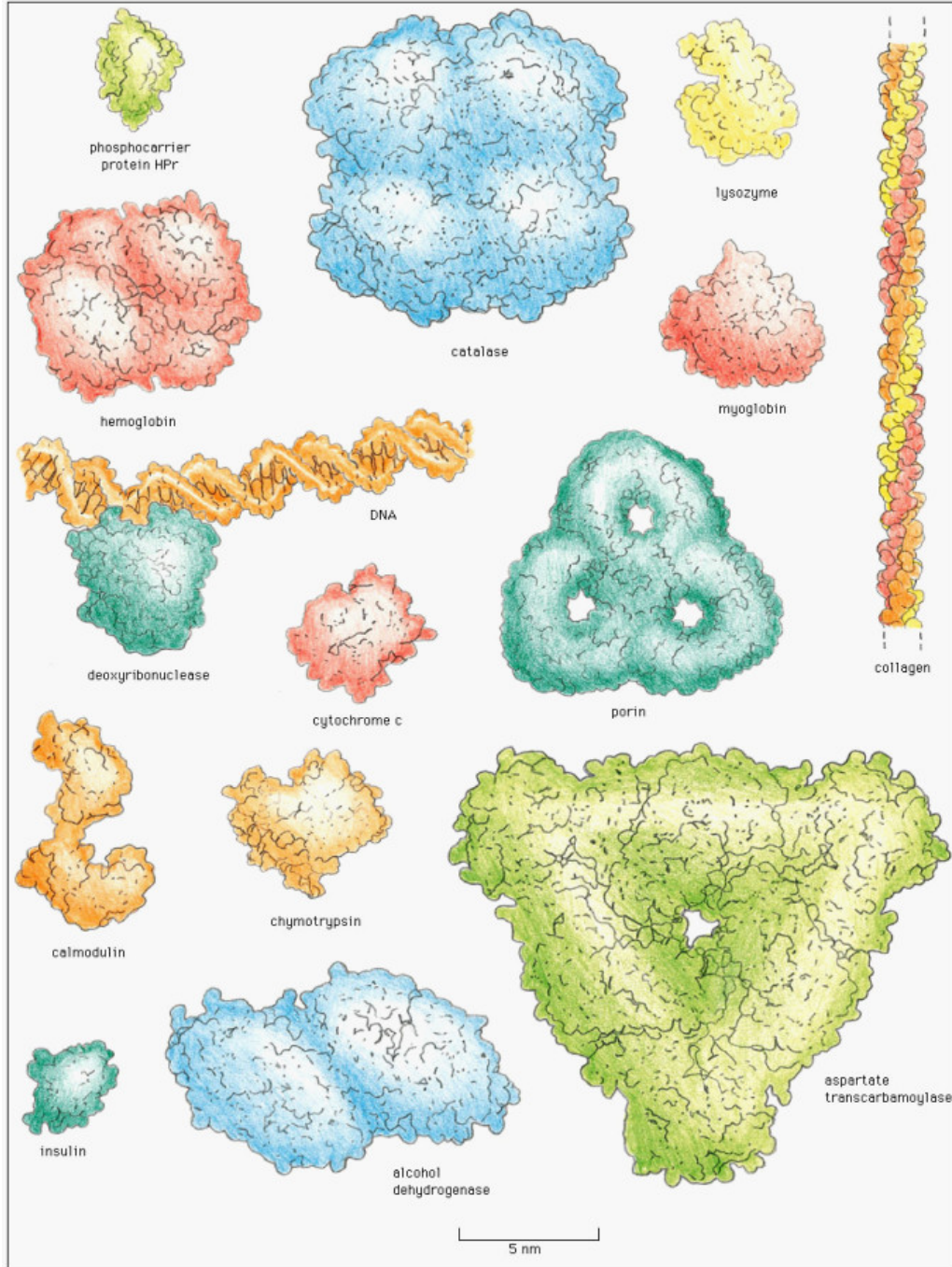


Frakcionisanje sirovog ekstrakta

- Osobine molekula globularnih proteina od značaja za njihovo izolovanje
- Frakciono taloženje
- Hromatografija:
 - gel filtracija
 - jonoizmenjivačka hromatografija
 - afinitetna hromatografija

Osobine molekula globularnih proteina
od značaja za njihovo izolovanje:

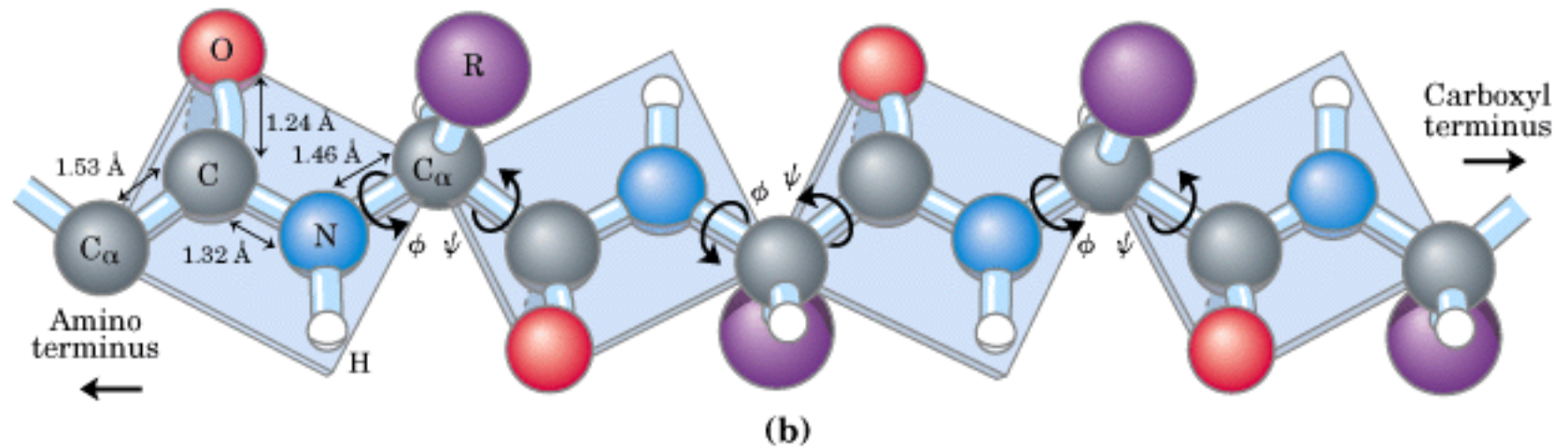
- Kako izgledaju molekuli proteina?
- Po kojim osobinama se molekuli proteina međusobno razlikuju?



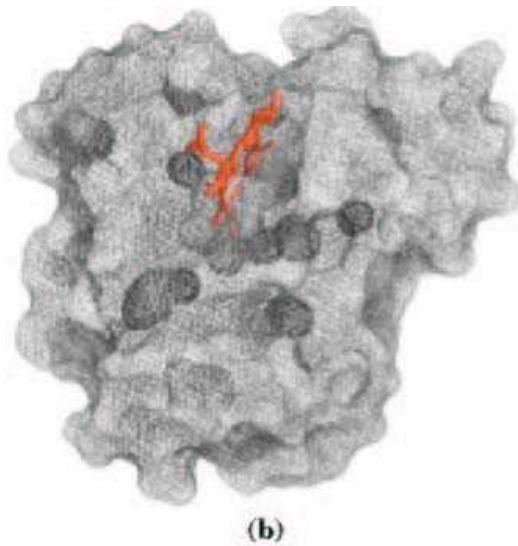
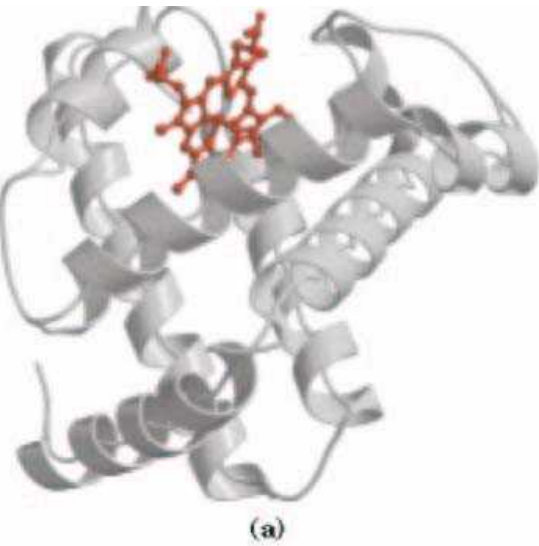
Kako izgledaju molekuli
proteina?

Globularni i fibrilni
proteini!

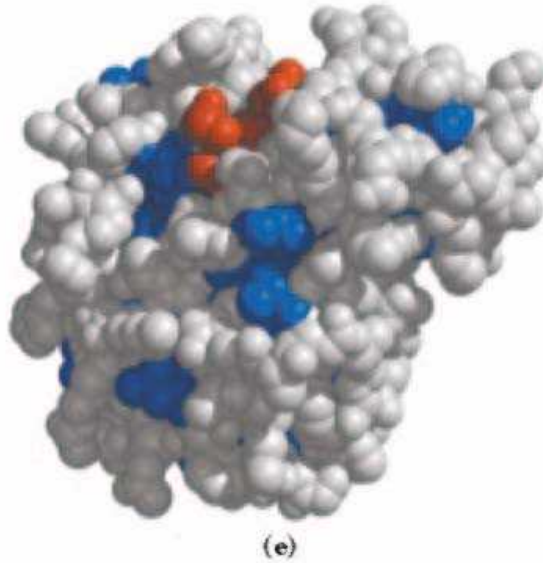
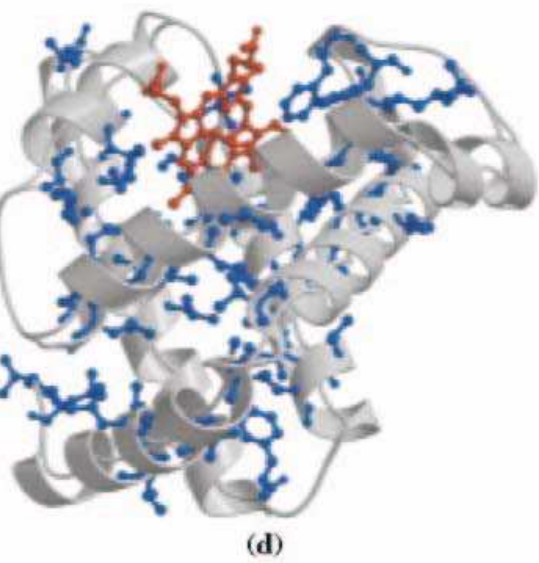
Istegnuti polipeptidni niz



Polipeptidni niz uvijen u nativnu globulu

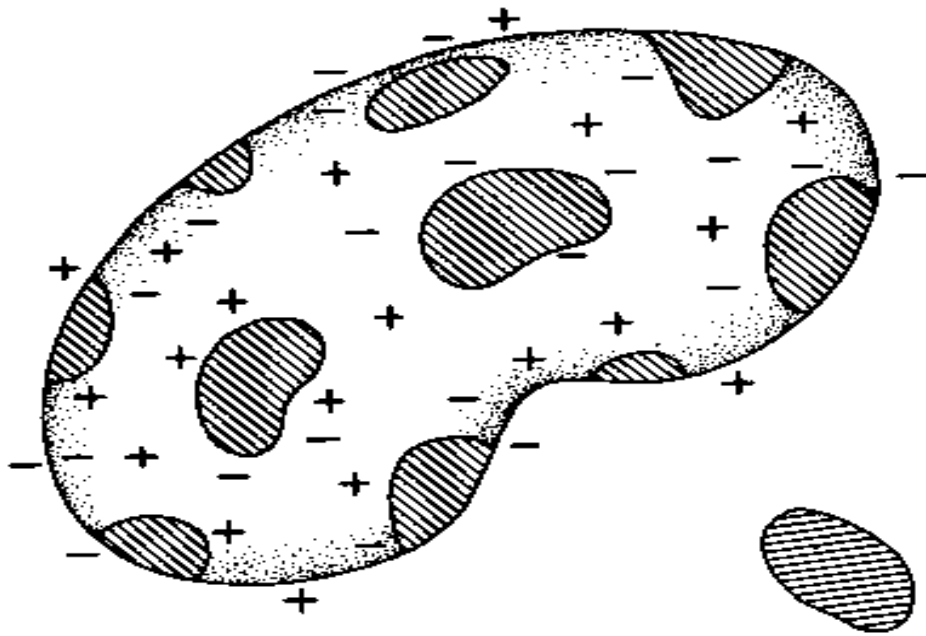


■ nepolarni ostaci



■ polarni ostaci

Po kojim osobinama se
molekuli proteina
međusobno razlikuju?



**Polarni/nepolarni ak
ostaci na površini:**
rastvorljivost

Veličina molekula: **gel
filtracija**

Naelektrisanje:
**jonoizmenjivačka
hromatografija**

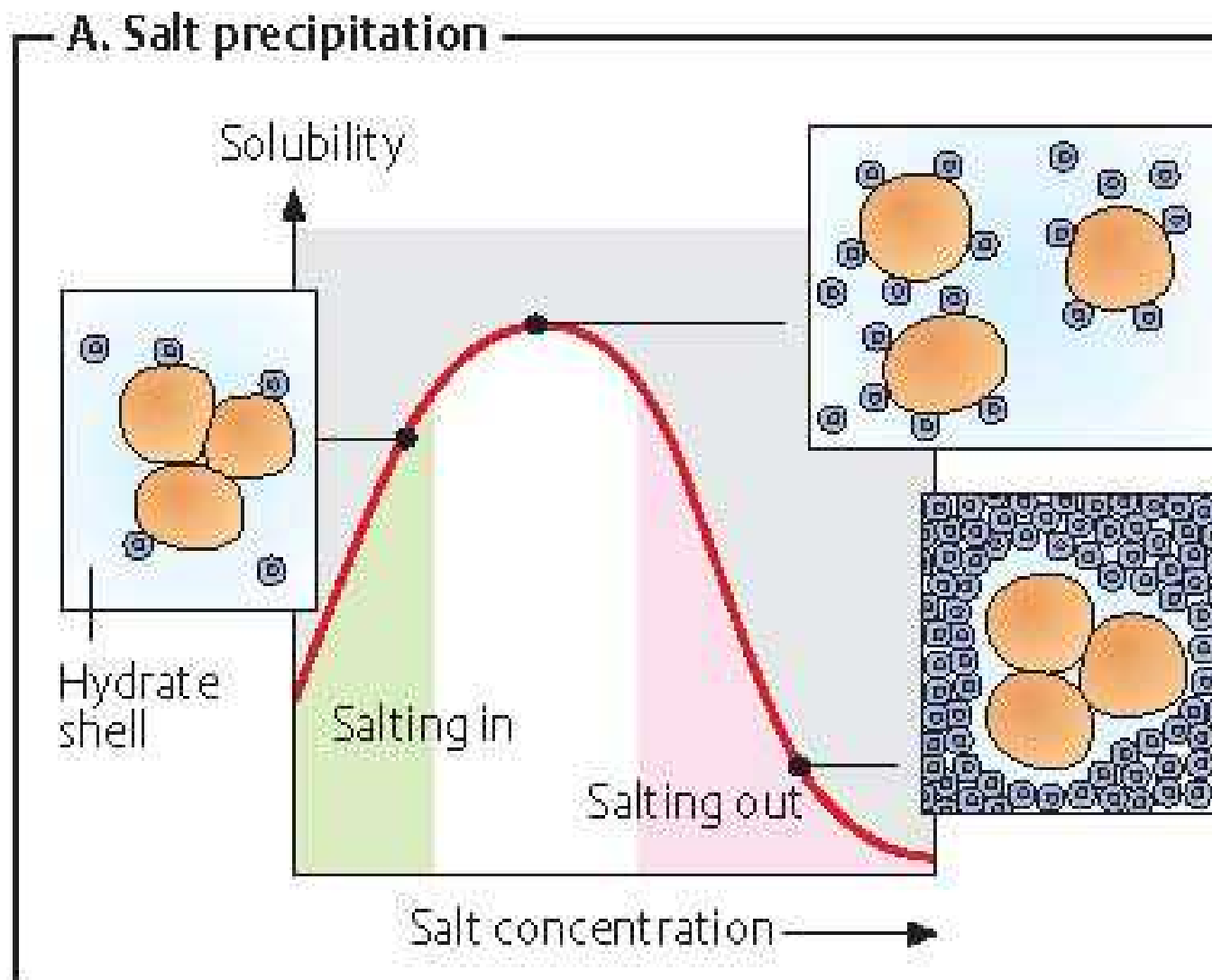
Aktivno mesto: **afinitetna
hromatografija**

(nepolarni) hidrofobni deo

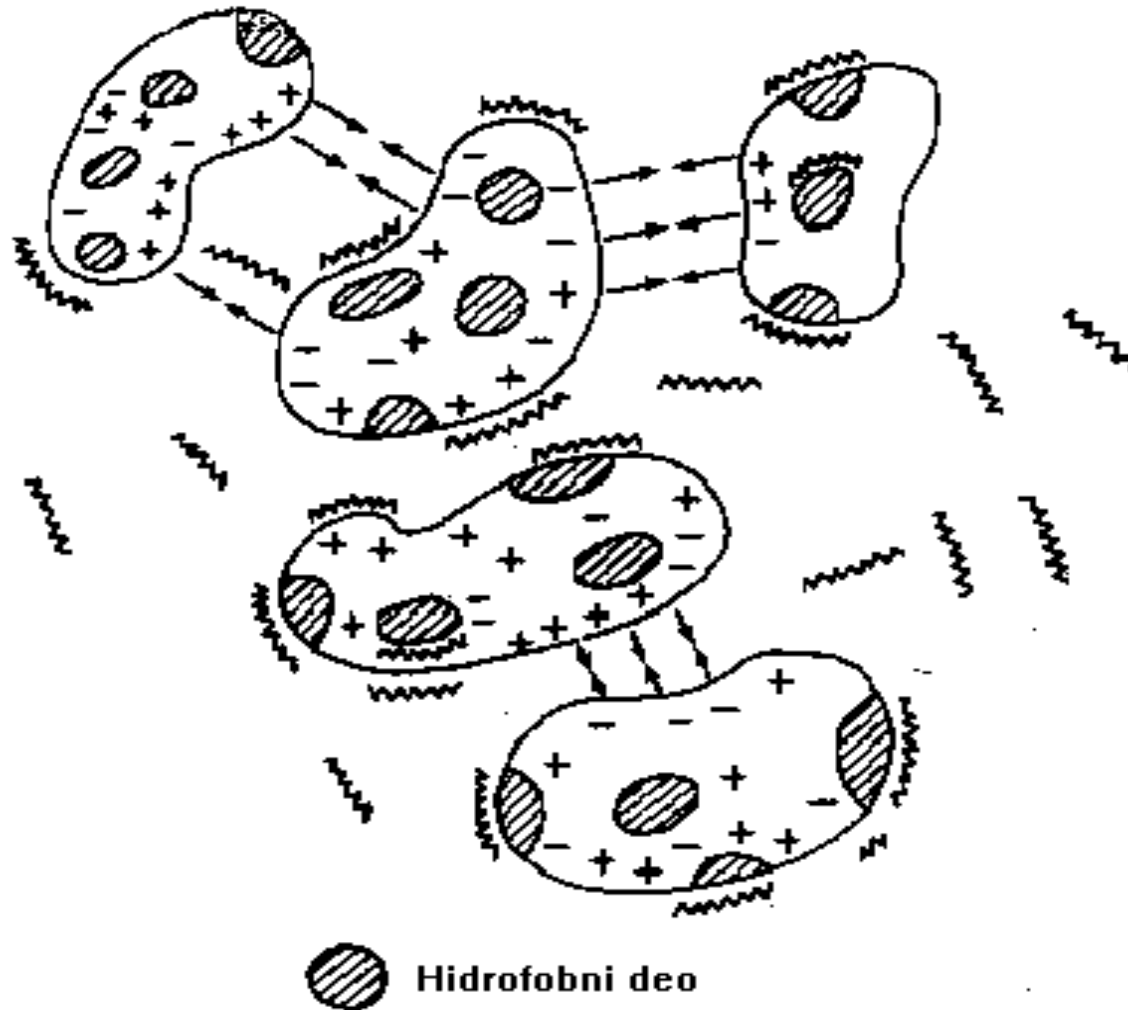
Frakciono taloženje

- Neorganske soli
 - Soli alkalnih metala *vs.* soli težkih metala
- Organski rastvarači
 - Mešljivi *vs.* nemešljivi sa vodom (efekat temperature!!!)
- Podešavanje pH na pI
- Povišena temperatura (manje stabilni proteini se denaturišu!!!)

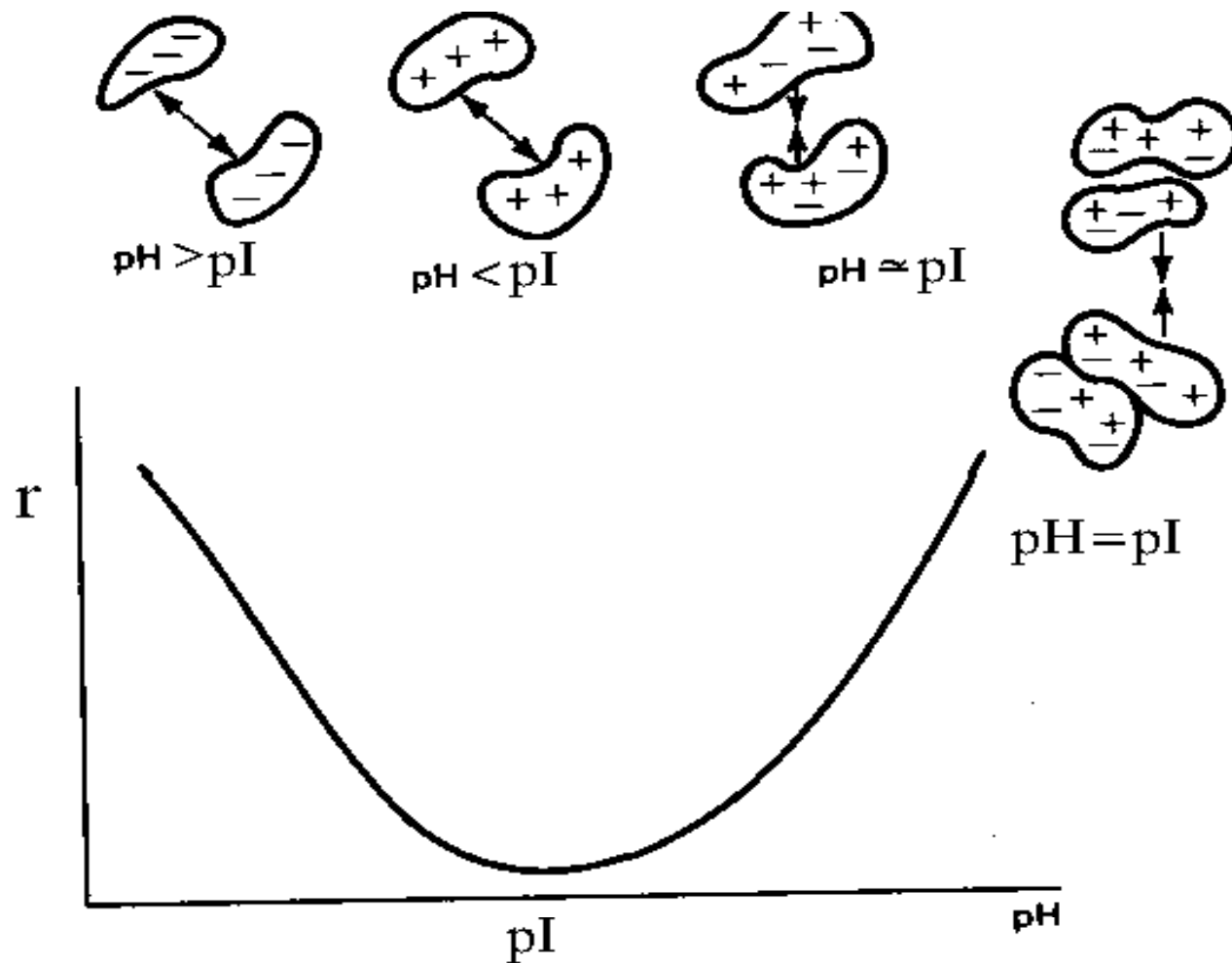
Taloženje solima



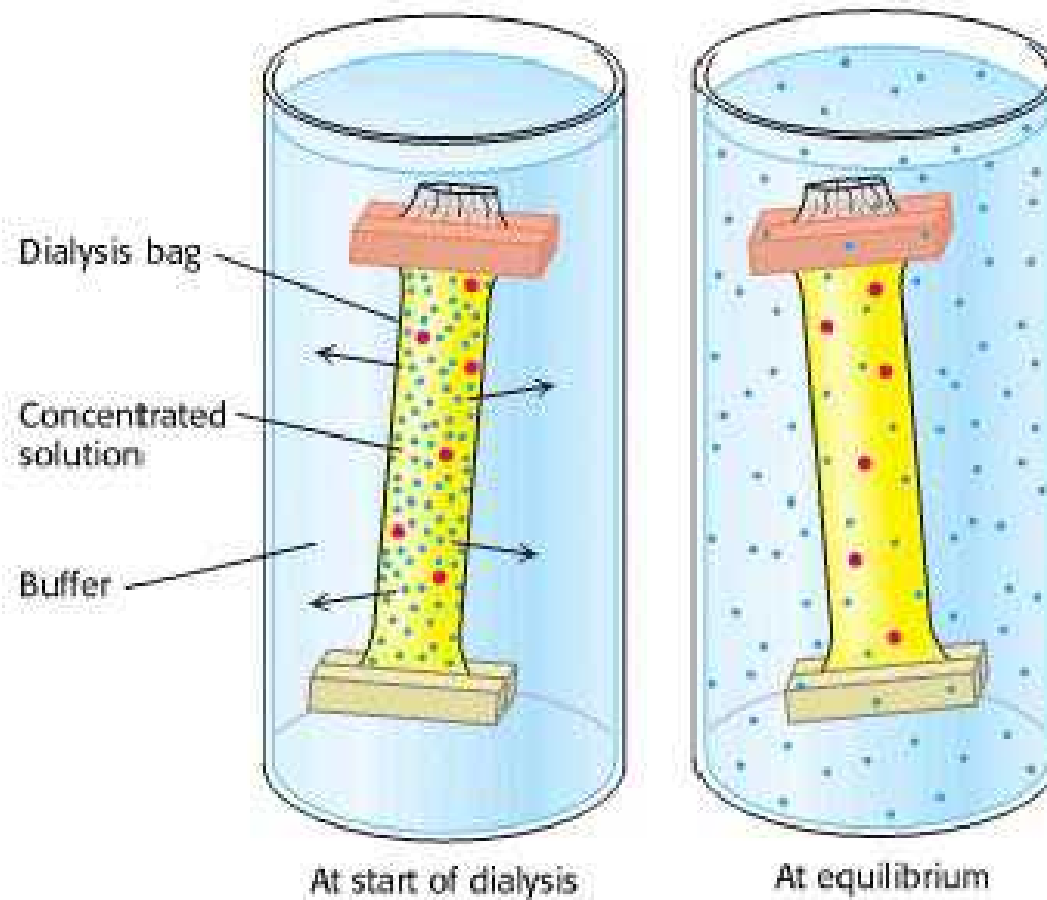
Taloženje organskim rastvaračima



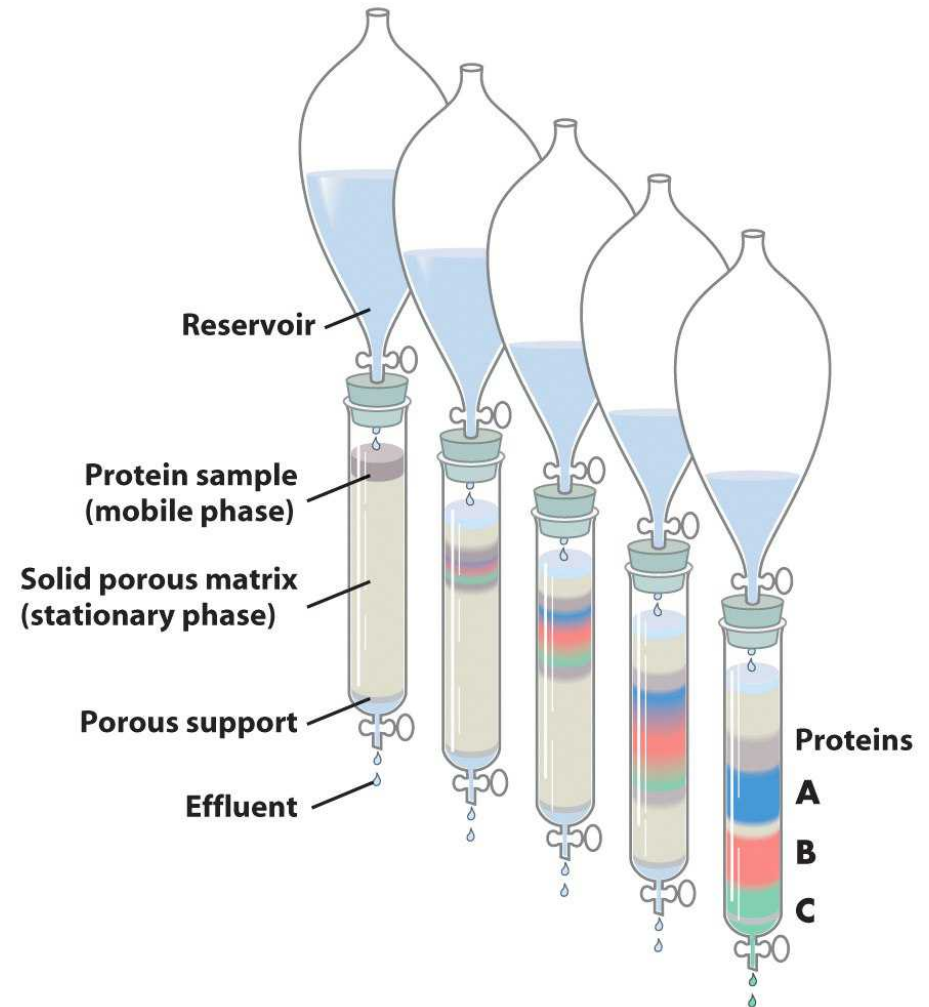
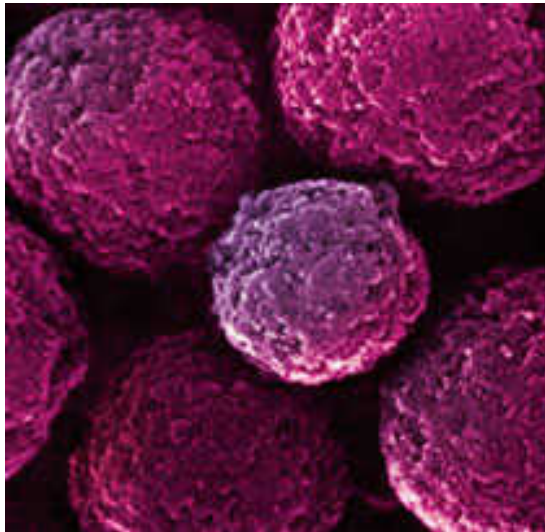
Taloženje podešavanjem pH na pI



Dijaliza: odvajanje proteina od malih molekula

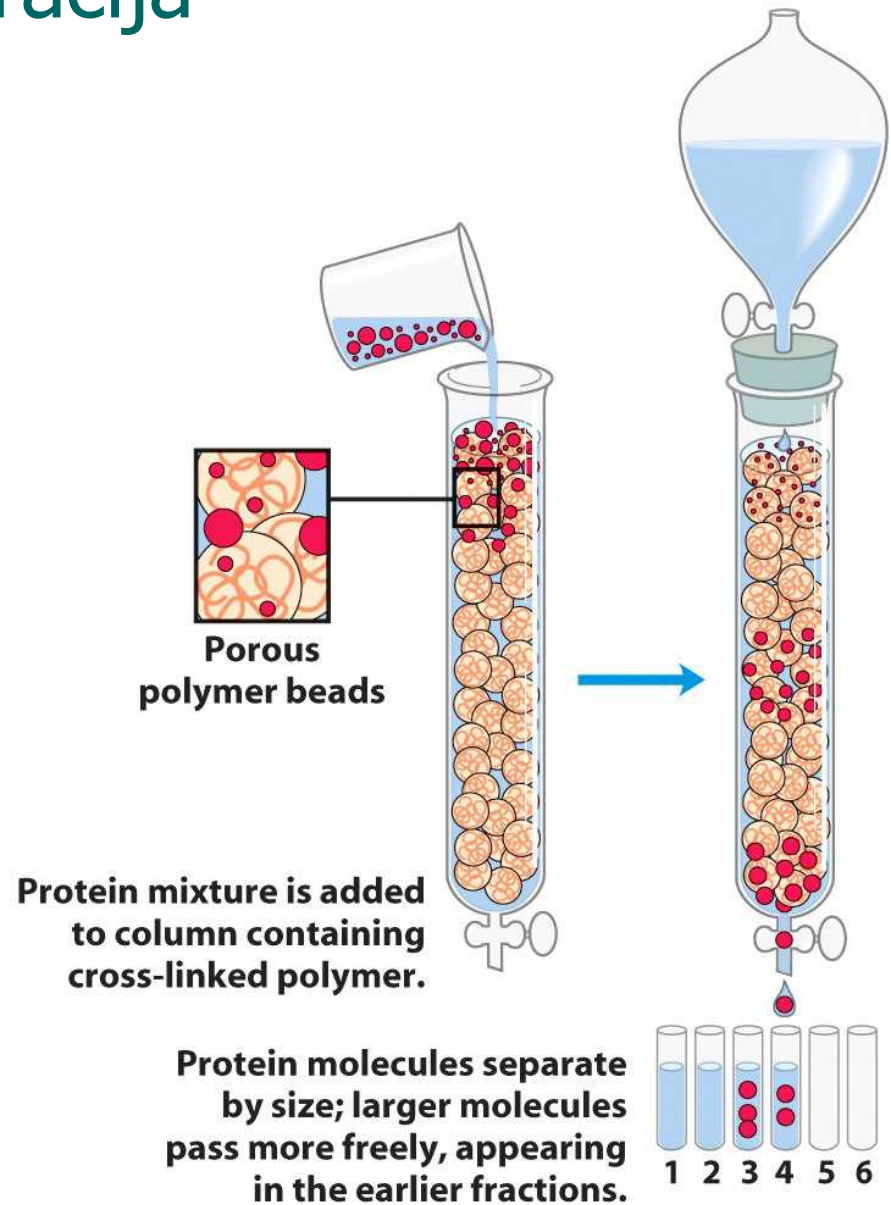


Hromatografske metode



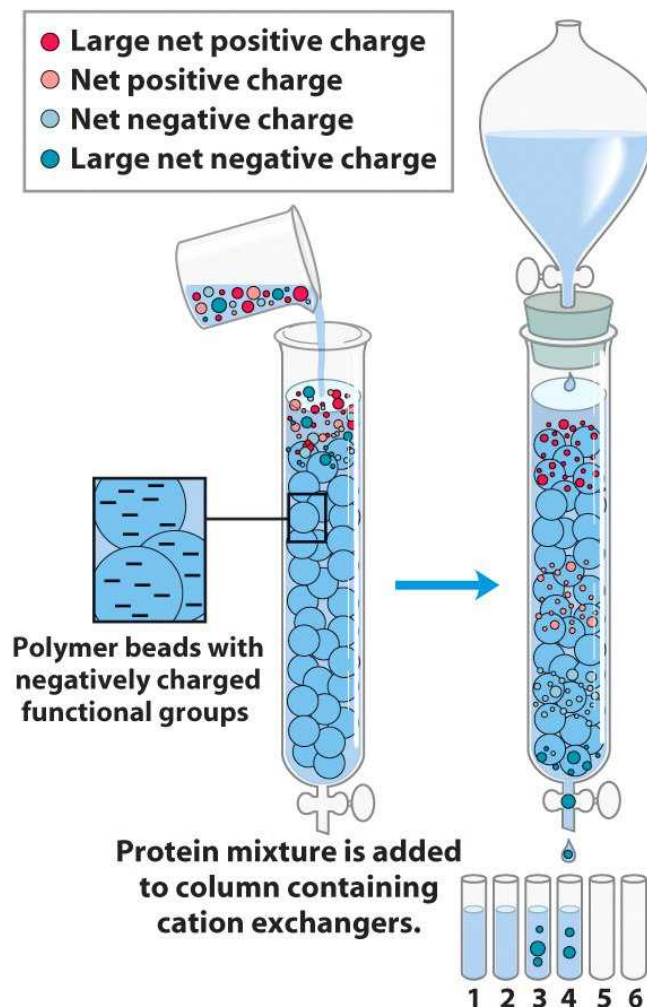
Gel filtracija

- Razdvajanje proteina na osnovu razlike u veličini/masi!
- Porozna zrna!



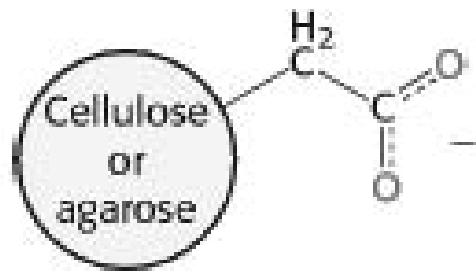
Jonoizmenjivačka hromatografija

- Razdvajanje proteina na osnovu razlike u naelektrisanju!
- pH vrednost!
- Jonska sila!
- Katjonska i anjonska jonoizmenjivačka hromatografija

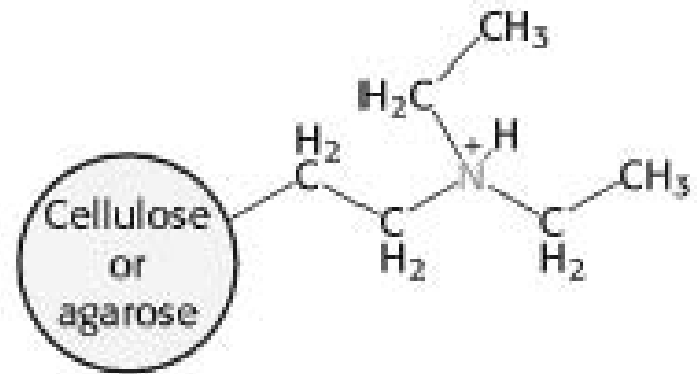


Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

Najčešće korišćeni jonoizmenjivači

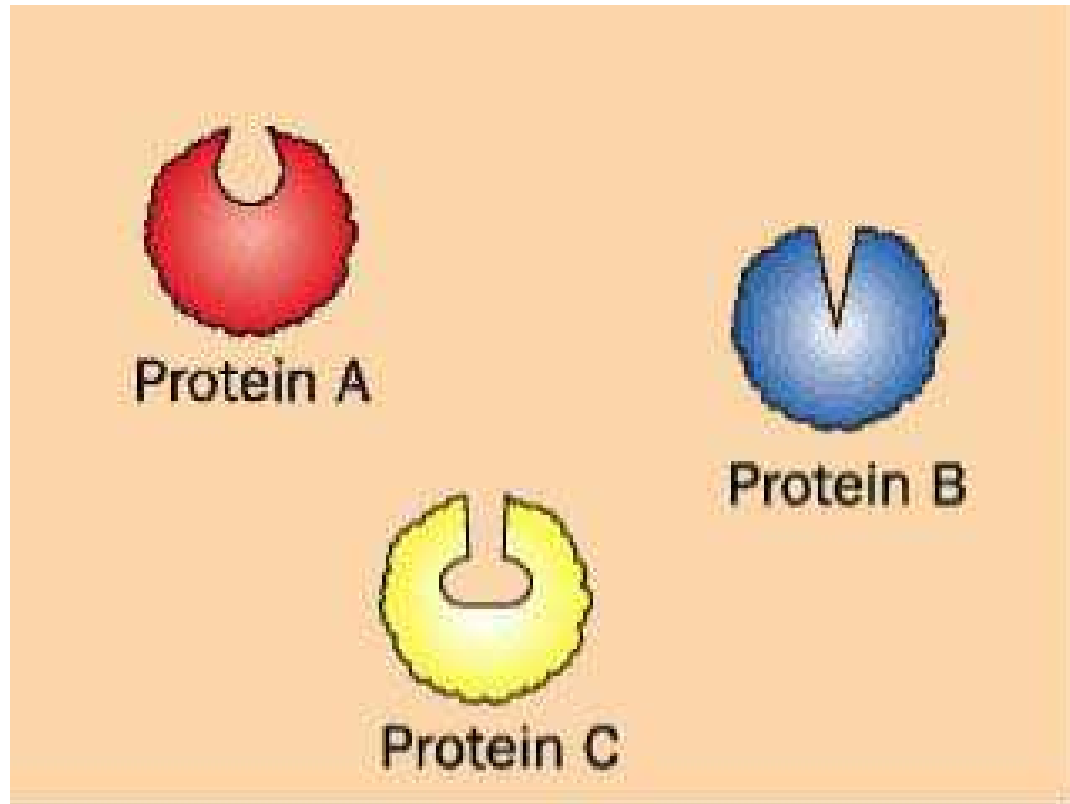


**Carboxymethyl
(CM) group**
(ionized form)



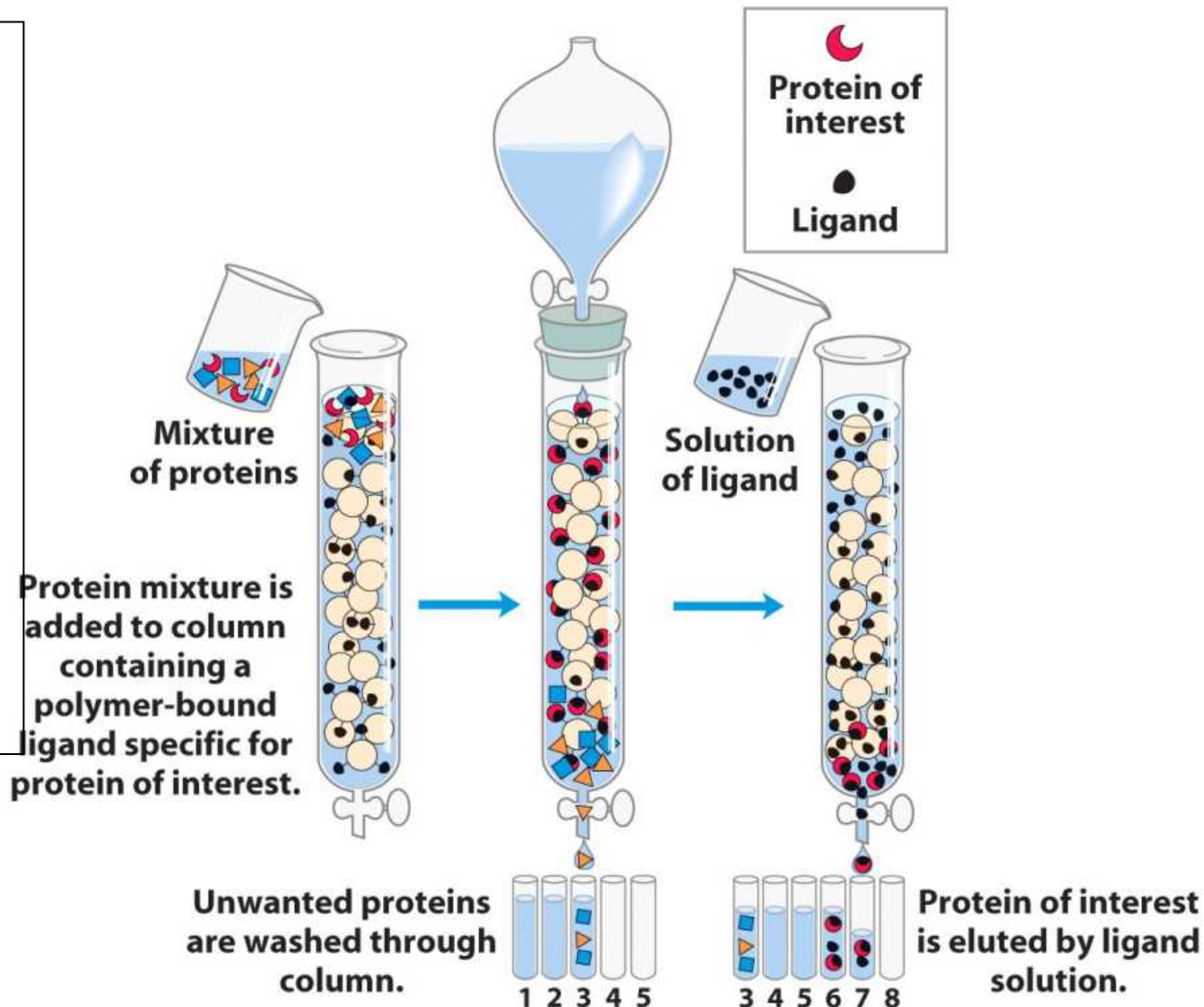
**Diethylaminoethyl
(DEAE) group**
(protonated form)

Afinitetna hromatografija

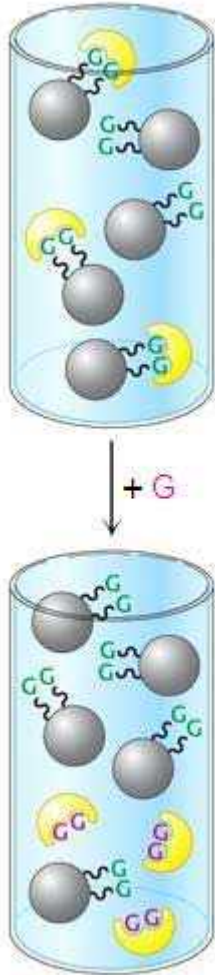


Afinitetna hromatografija

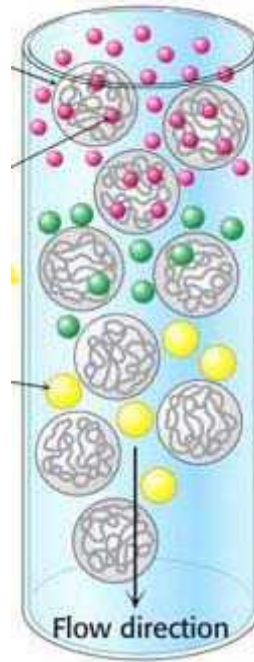
- Razdvajanje proteina na osnovu specifičnih interakcija!
- Biološki parovi!
 - enzim-supstrat
 - receptor-ligand
 - antitelo-antigen



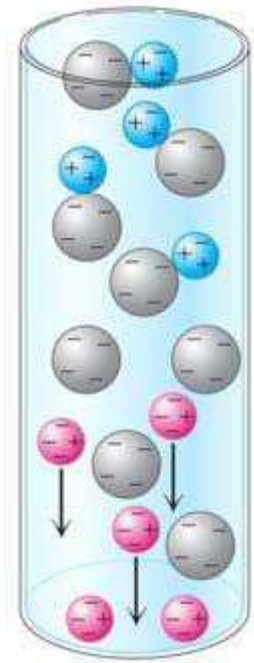
A?



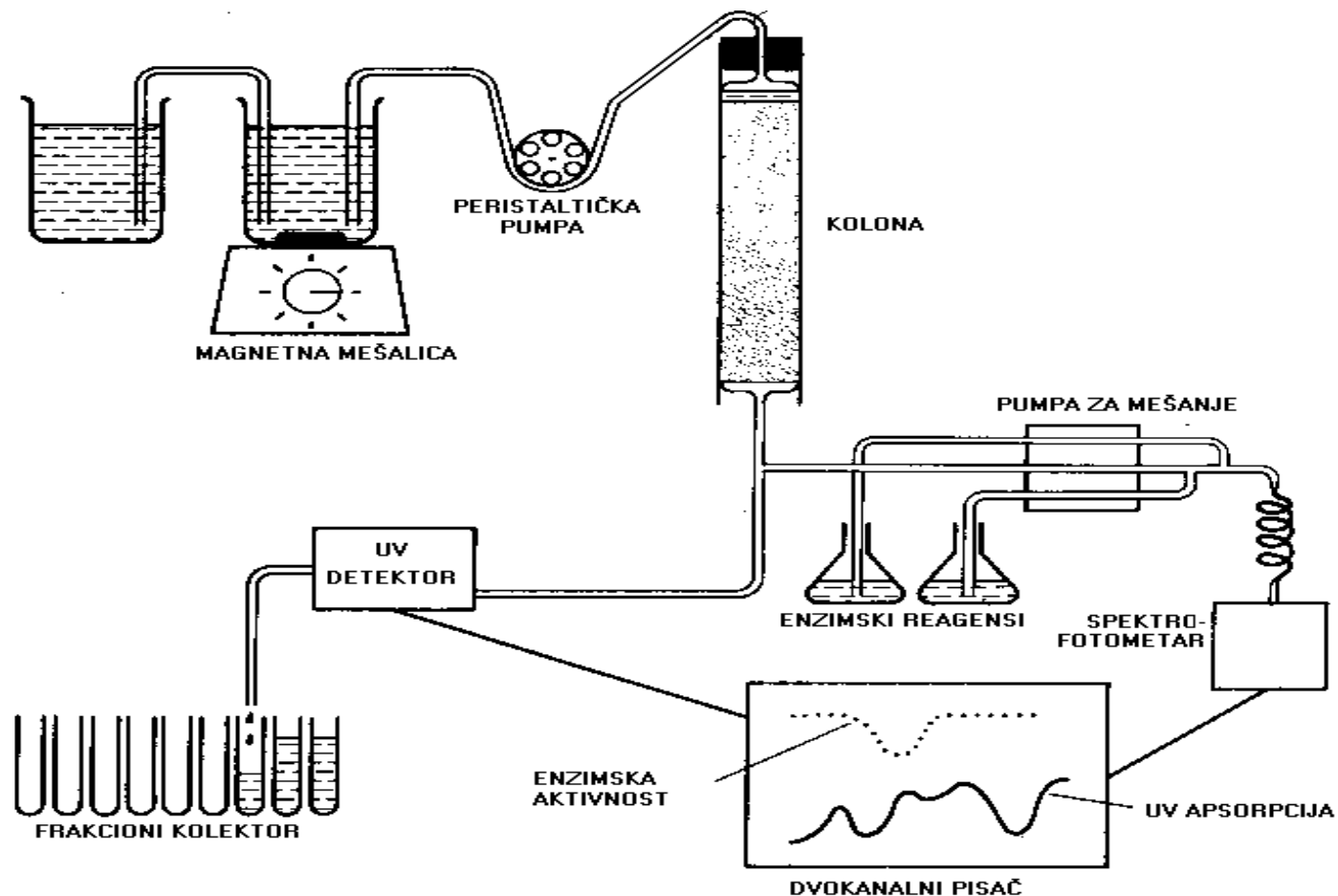
B?



C?



Hromatografske metode

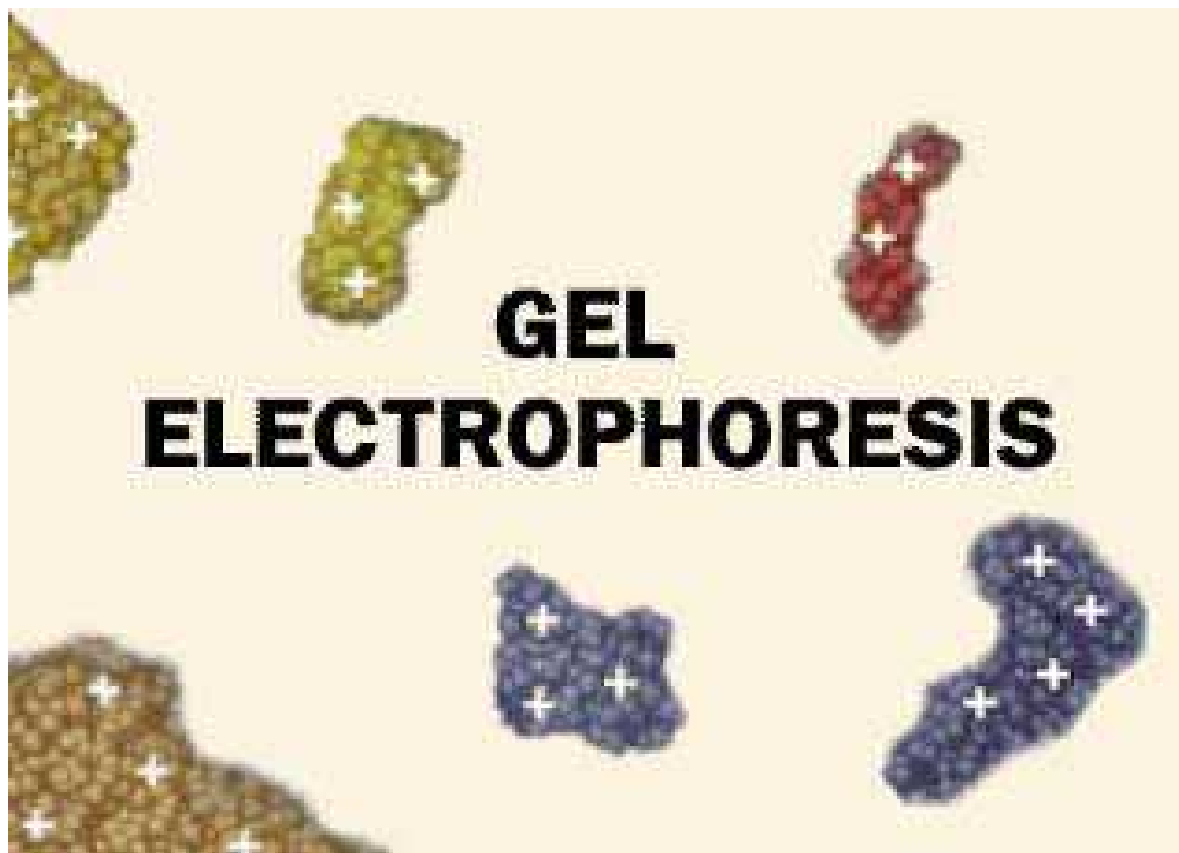


Aparatura za hromatografiju proteina

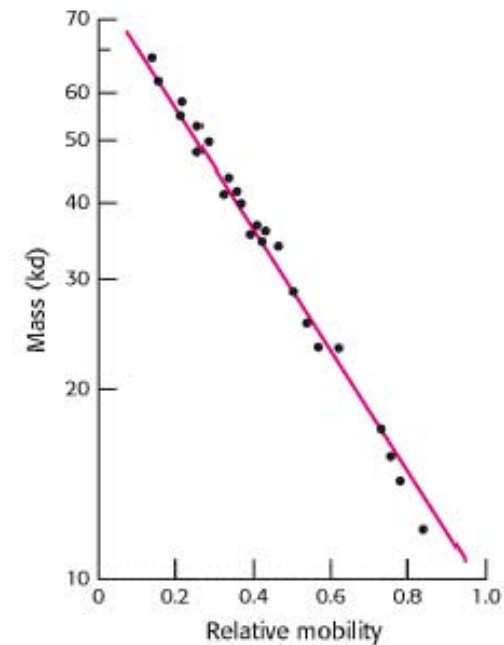
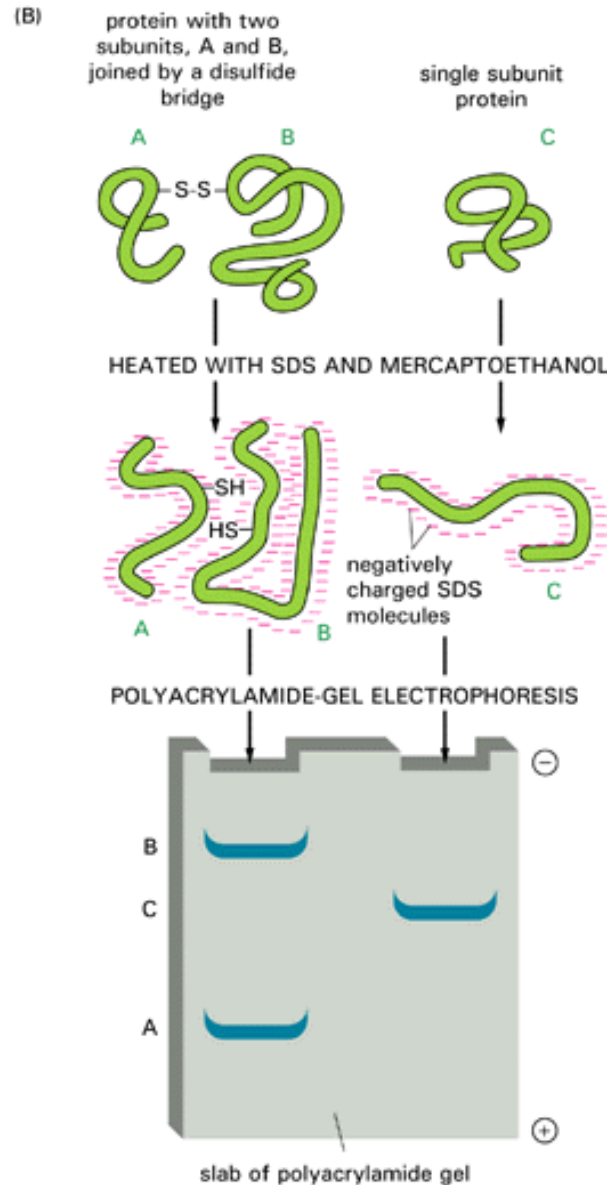
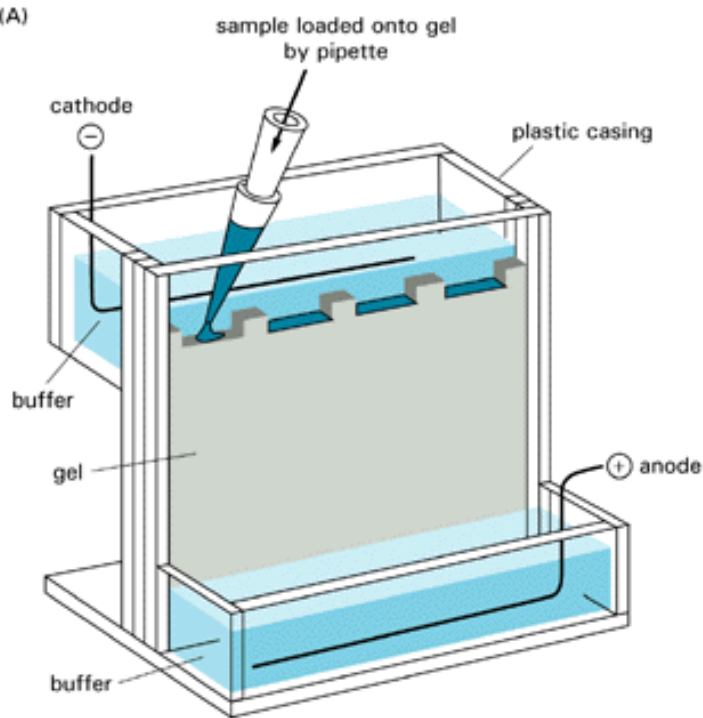
Provera homogenosti (čistoće):

- elektroforetske tehnike
- izoelektrično fokusiranje
- 2D elektroforeza

Elektroforetske tehnike

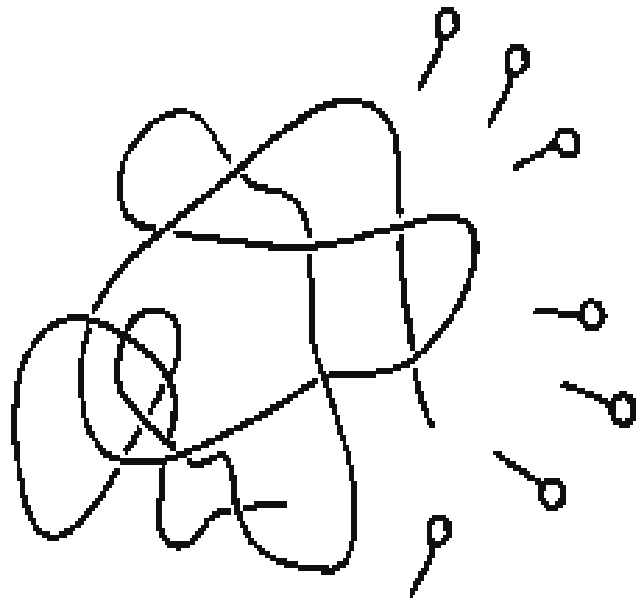


SDS elektroforeza

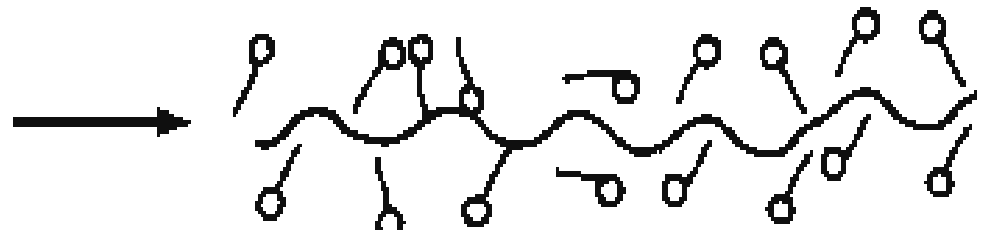


Vezivanje SDS-a za molekul proteina

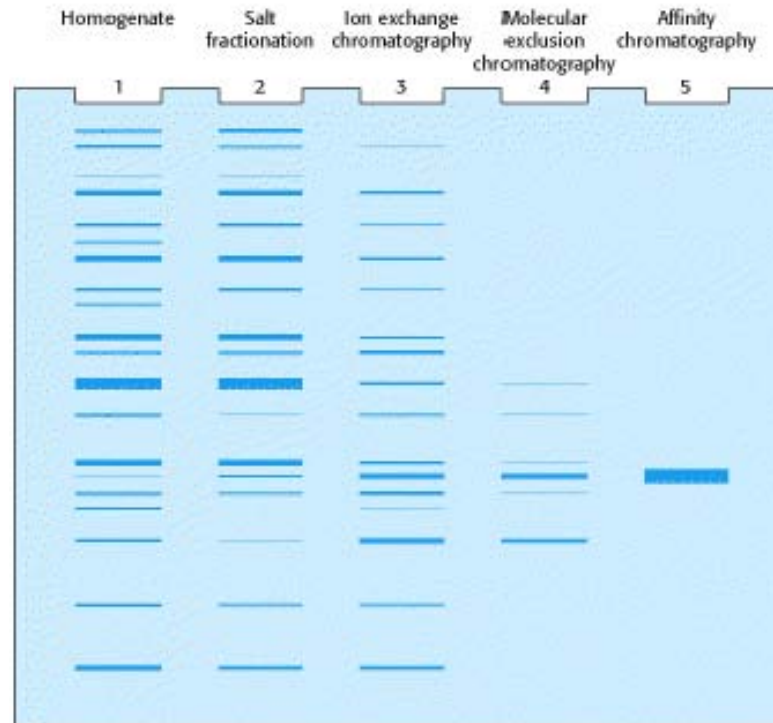
Nativni protein + SDS



Denaturisani polipeptid u formi negativno naelektrisanog štapića

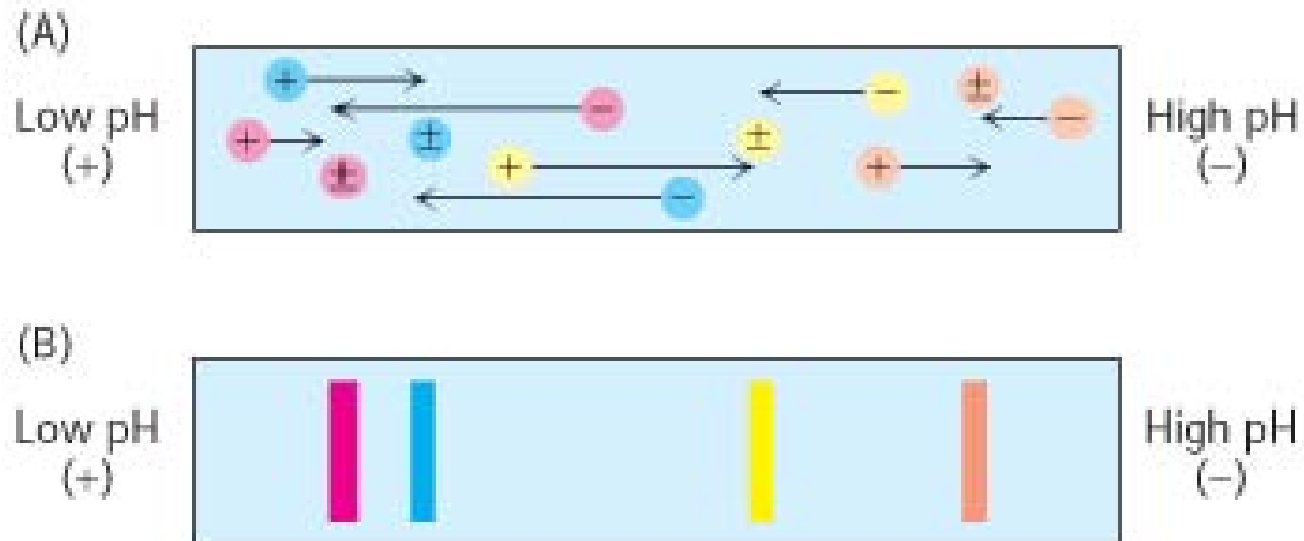


Primer izolovanja proteina



Step	Total protein (mg)	Total activity (units)	Specific activity, (units mg ⁻¹)	Yield (%)	Purification level
Homogenization	15,000	150,000	10	100	1
Salt fractionation	4,600	138,000	30	92	3
Ion-exchange chromatography	1,278	115,500	90	77	9
Molecular exclusion chromatography	68.8	75,000	1,100	50	110
Affinity chromatography	1.75	52,500	30,000	35	3,000

Princip izoelektričnog fokusiranja



pH gradient se uspostavi pre nanošenja uzorka. (A) Nanese se uzorak i uključi napon. Proteini se kreću dok ne dođu do $\text{pH} = \text{pI}$ i tu daju trake koje se mogu videti nakon bojenja gela (B).

Dvodimenzionalna, 2D elektroforeza

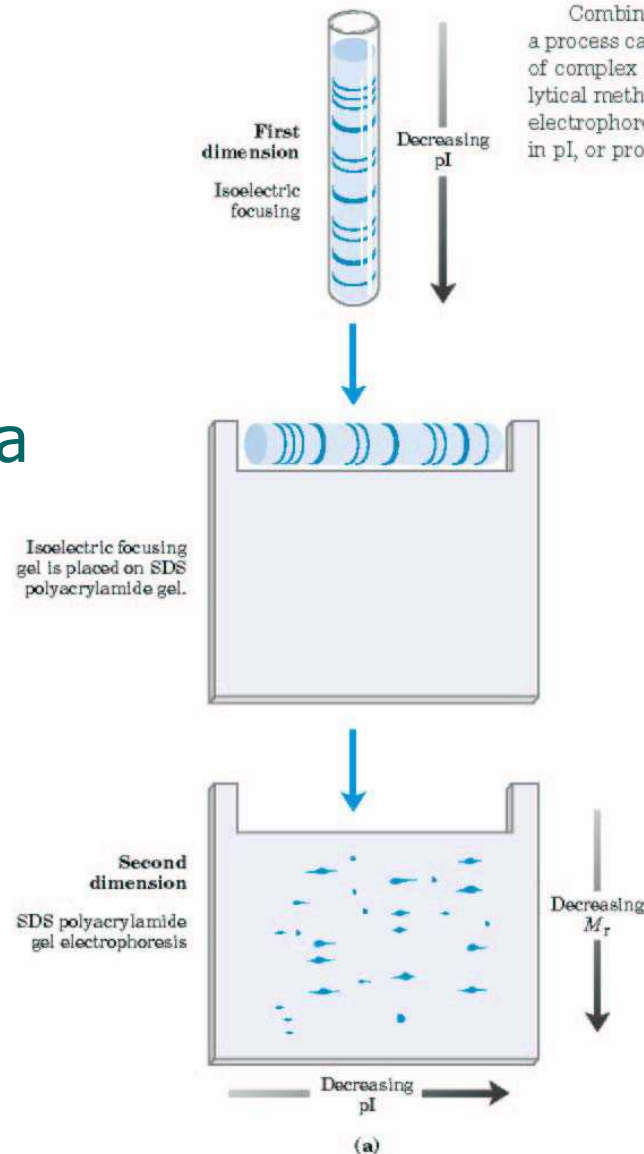
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Part II Structure and Catalysis

- Izoelektrično fokusiranje u jednom smeru,
- SDS elektroforeza u drugom smeru

Primena:

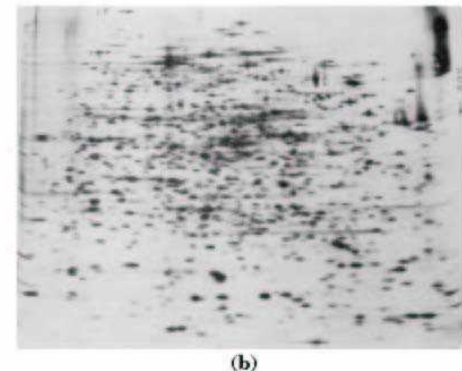
- ispitivanje homogenosti
- proteom(iks)!



Combining isoelectric focusing and SDS electrophoresis sequentially in a process called **two-dimensional electrophoresis** permits the resolution of complex mixtures of proteins (Fig. 5-22). This is a more sensitive analytical method than either electrophoretic method alone. Two-dimensional electrophoresis separates proteins of identical molecular weight that differ in pI, or proteins with similar pI values but different molecular weights.

figure 5-22

Two-dimensional electrophoresis. (a) Proteins are first separated by isoelectric focusing in a cylindrical gel. The gel is then laid horizontally on a second, slab-shaped gel, and the proteins are separated by SDS polyacrylamide gel electrophoresis. Horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight. (b) More than 1,000 different proteins from *E. coli* can be resolved using this technique.



Genom i proteom

- Sekvenca DNK mnogih organizama je određena!!!
- Humani genom sadrži oko 3 milijarde baza i oko 40 000 gena.
- **Proteom** (exprimirani protein) je funkcionalna reprezentacija **genoma**.
- Razumevanje proteoma postizemo ispitivanjem, karakterisanjem, katalogiziranjem pojedinačnih proteina.
- Istraživač obično započinje proces odvajanjem određenog proteina od svih drugih biomolekula u ćeliji.

Karakterisanje proteina

Ispitivanje i (evt) primena izolovanog proteina

Karakterisanje:

- molekulska masa, pI, aminoanaliza, aminokiselinska sekvenca (nalaženje evolucionih odnosa sa drugim poznatim proteinima), određivanje aktivnosti...

Ispitivanje:

- 3D struktura
- odnos strukture i aktivnosti...

Primena.....