

CORSO DI LAUREA IN BIOLOGIA PER LA SOSTENIBILITÀ



BIOCHIMICA APPLICATA (6 CFU)

LEZIONE 7

Prof. Paola Di Donato

Dipartimento di Scienze e Tecnologie

Stanza 520, V piano lato NORD

Tel. 081 547 6625

E-mail: paola.didonato@uniparthenope.it

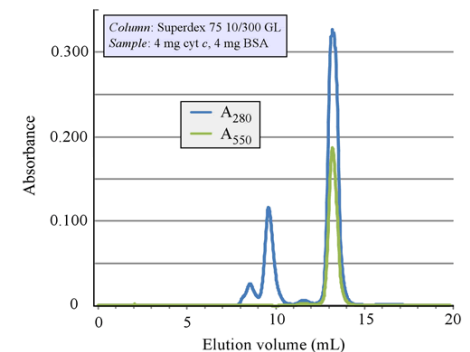
Tecniche per lo studio e la caratterizzazione delle proteine

Determinazione massa molecolare; numero e peso molecolare delle subunità

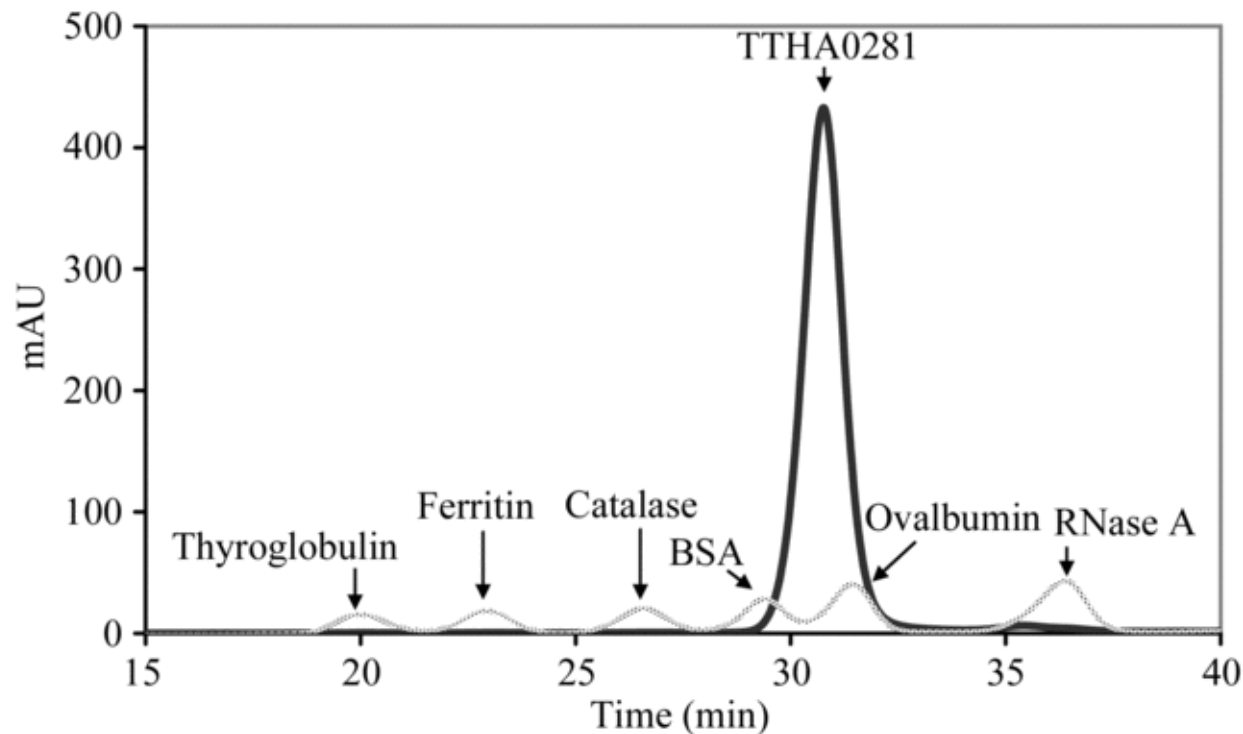
- ✓ Cromatografia per esclusione molecolare
- ✓ SDS-PAGE
- ✓ Ultracentrifugazione analitica
- ✓ Spettrometria di massa
- ✓ Light scattering

Determinazione massa molecolare; numero e peso molecolare delle subunità

✓ Cromatografia per esclusione molecolare



Size-exclusion chromatography analysis of the recombinant TTHA0281 protein

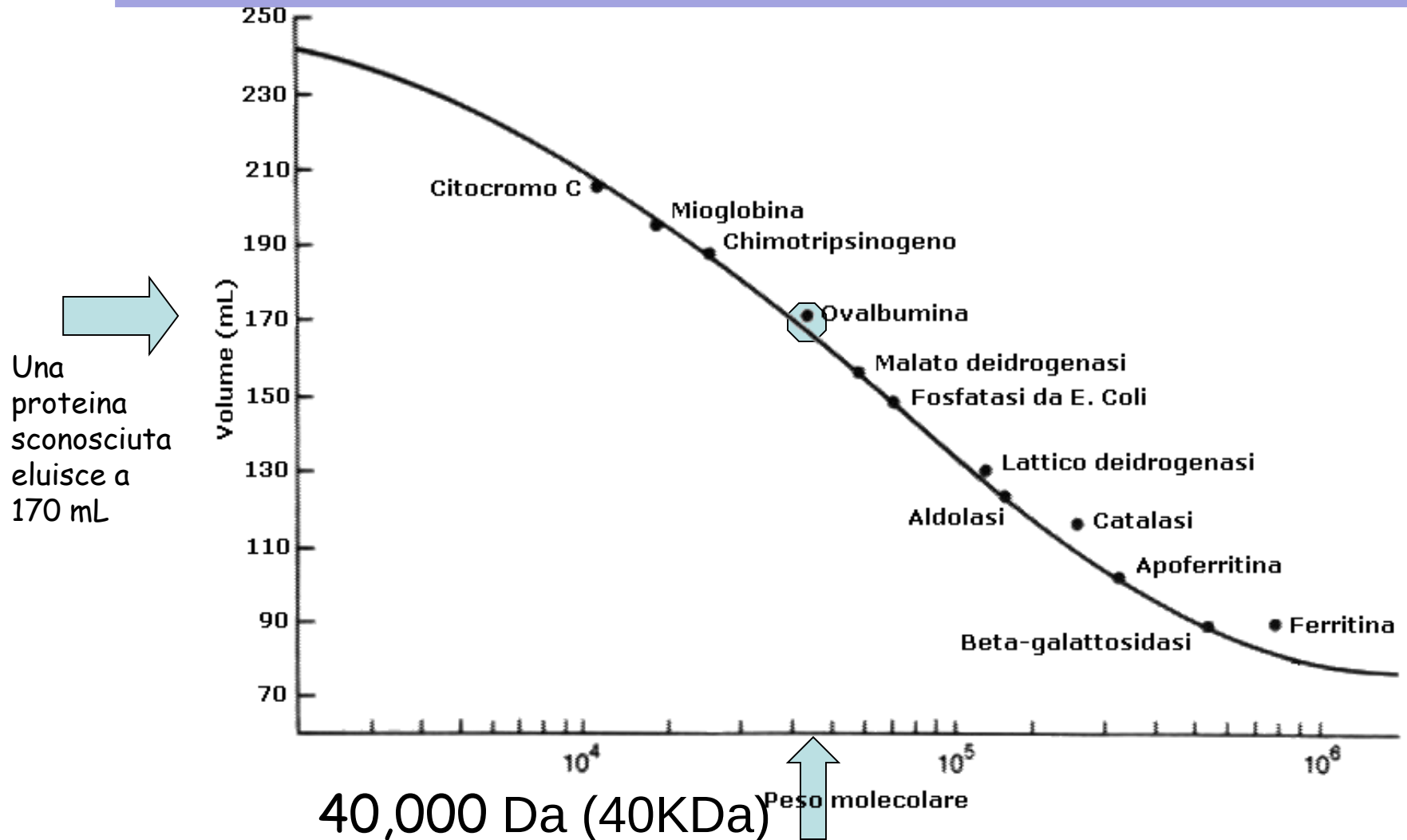


The purified TTHA0281 protein (1.9 $\mu\text{g}/0.2 \mu\text{l}$, black line) and 0.6 μl molecular-weight standards (grey line), containing 0.2 μg thyroglobulin (669 kDa), 0.4 μg ferritin (440 kDa), 0.1 μg catalase (158 kDa) and 0.3 μg each of RNase A (13.7 kDa), ovalbumin (43 kDa) and bovine serum albumin (BSA) (67 kDa),

The elution profile of the sample was detected by measuring A_{215} .

"Structure of a UPF0150-family protein from Thermus thermophilus HB8" Acta Crystallogr Sect F Struct Biol Cryst Commun. 2007;63:173-7. Okazaki N et al.

La filtrazione su gel può essere usata per stimare la massa molecolare



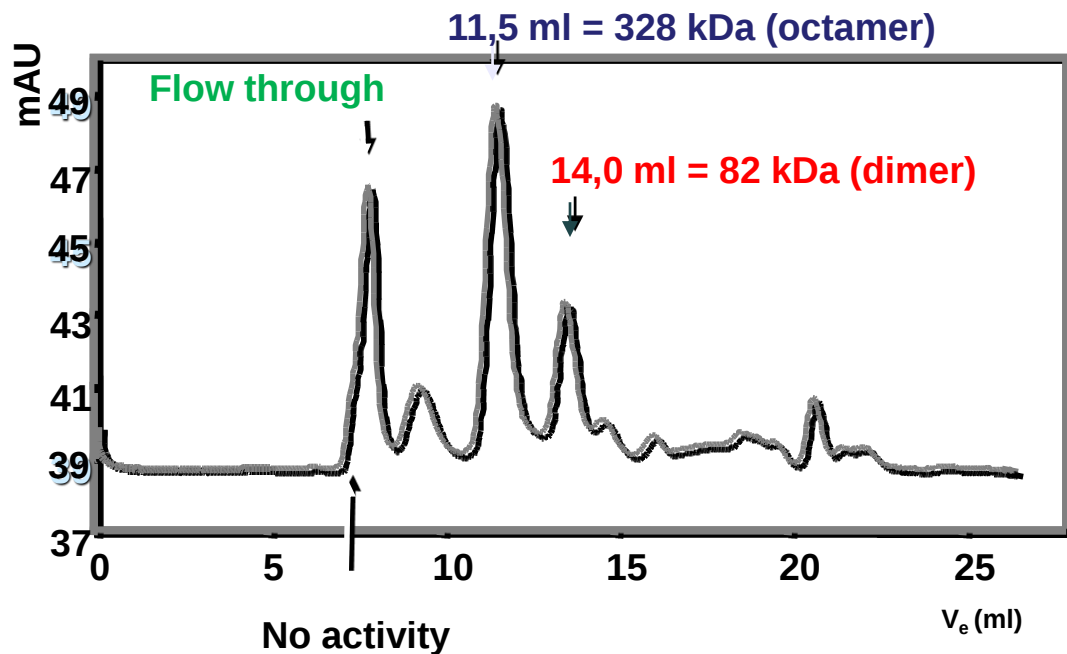
Volume di eluizione relativo verso il logaritmo della massa molecolare.



Quaternary structure of recombinant SsoGMPP



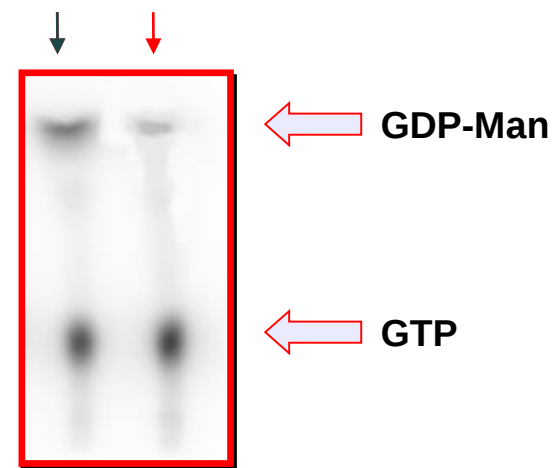
Apparent molecular weight analysis by gel filtration on Superdex-200 column



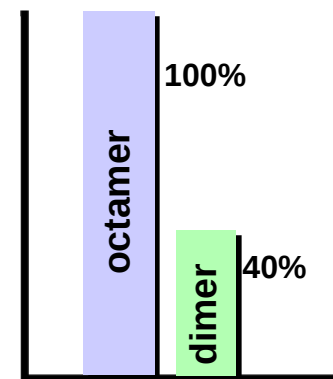
Recombinant SsoGMPP presents two quaternary structures: an octameric and a dimeric form (with an apparent molecular weight respectively of 328 and 82 kDa), with the octamer showing higher activity.

Radioactive assay

11,5 ml 14,0 ml



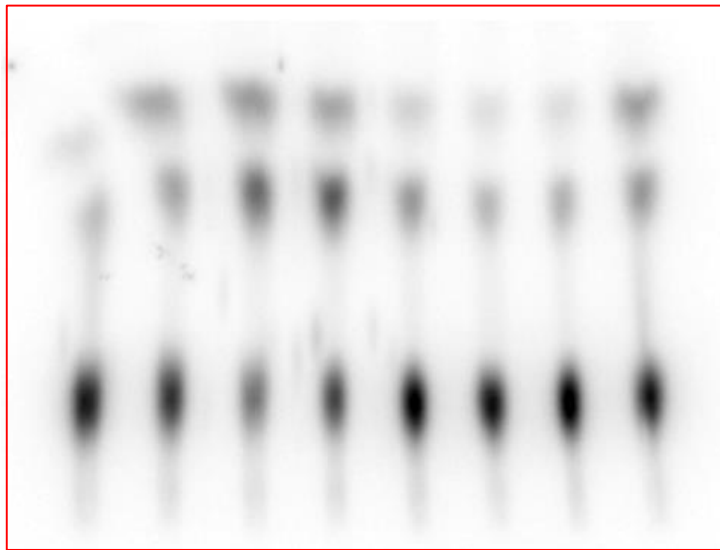
Specific activity (%)



Gel-filtrazione su S-200 di omogenato di *S. solfataricus*

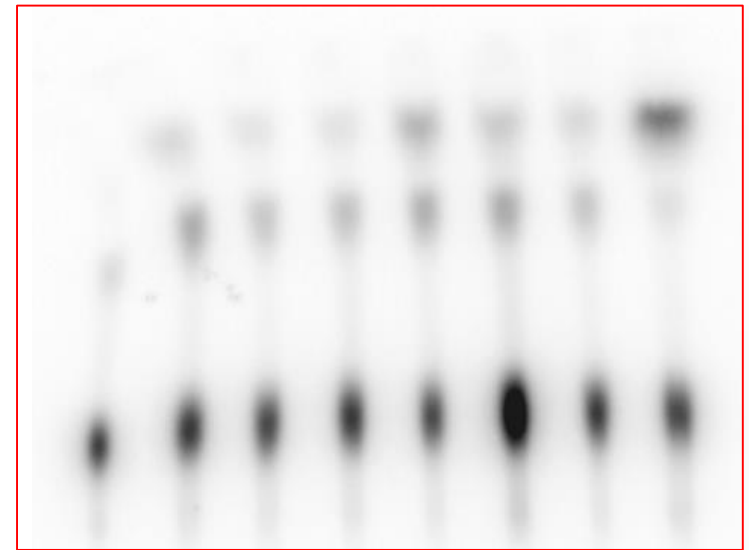
Rivelato per attività GDP-mannosio pirofosforilasi (saggio radioattivo) e analizzato su TLC e autoradiografia

GDP-mannosio
→



21 22 23 24 25 26 27 28

Ottamero di
328 kDa



B
S
A 25 26 27 28 29 30 S

Dimero di
82 kDa

S: SsoGMPP ricombinante

Studio della struttura delle proteine: applicazioni della gel filtrazione

“Despite its high similarity with monomeric arginine kinase, muscle **creatine kinase** is only enzymatically active as a dimer”

Awama, Mazon, Vial, Marcillat

Archives of Biochemistry and Biophysics 458 (2007) 158-166

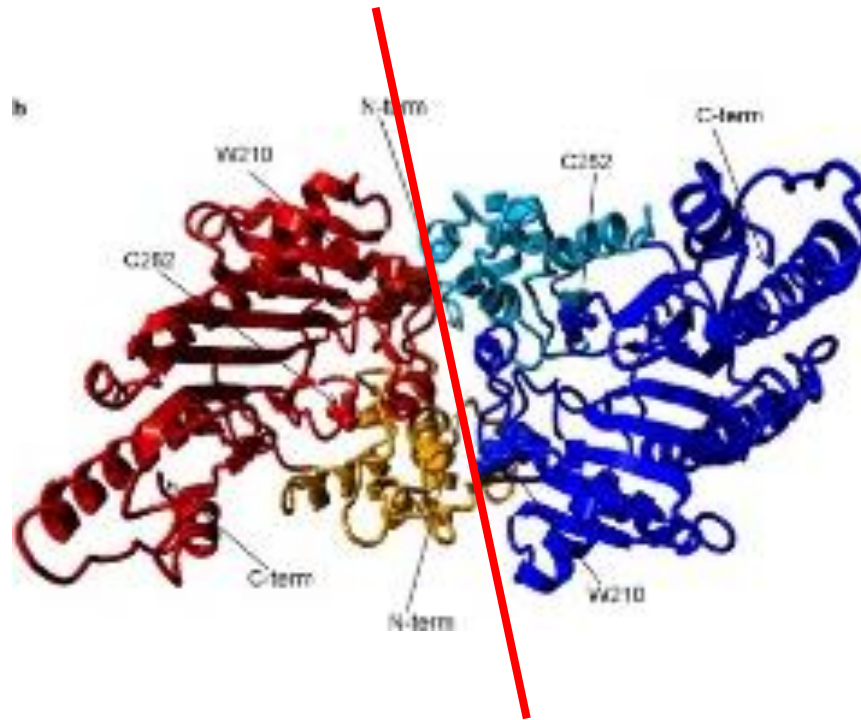
Studio della struttura delle proteine: l'esempio della creatina chinasi (CK)



- L'attività catalitica della CK dipende dalla struttura quaternaria: essa è attiva in forma dimerica, mentre le singole subunità non hanno attività apprezzabile
- La CK del muscolo è un dimerico le cui subunità sono associate da interazioni elettrostatiche all'interfaccia tra le catene
- L'aggiunta di NaCl e cloruro di Guanidinio comporta la distruzione delle interazioni all'interfaccia, dunque la dissociazione dei monomeri e la conseguente diminuzione dell'attività catalitica
- Tale comportamento è stato confermato mediante l'uso della cromatografia di esclusione

Studio della struttura delle proteine: l'esempio della creatina chinasi (CK)

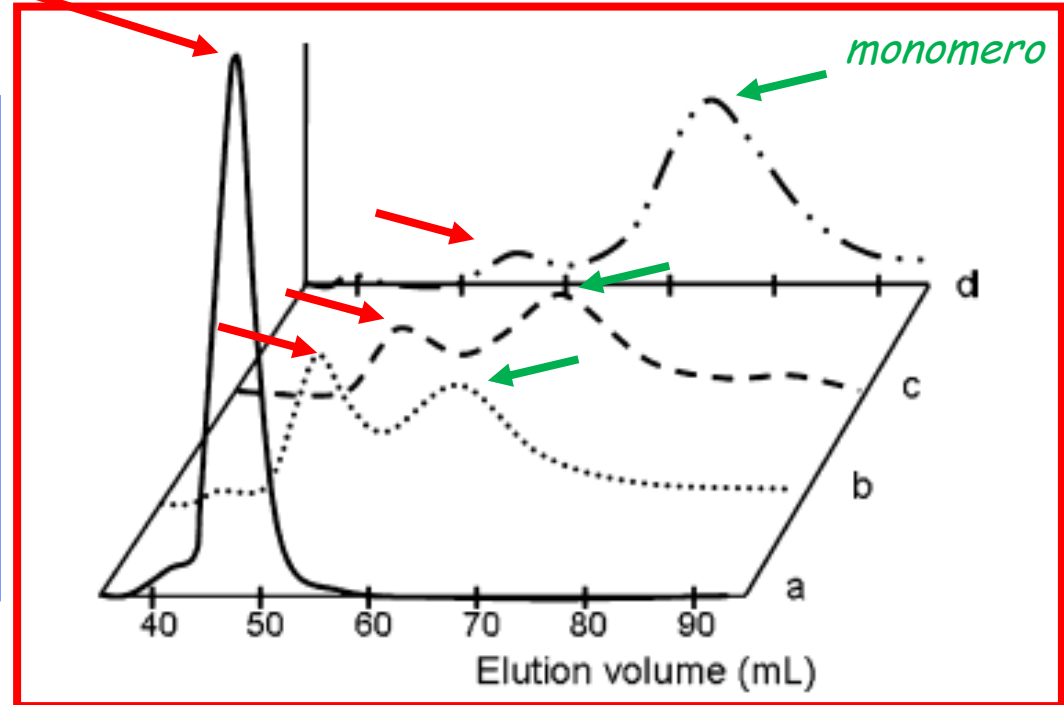
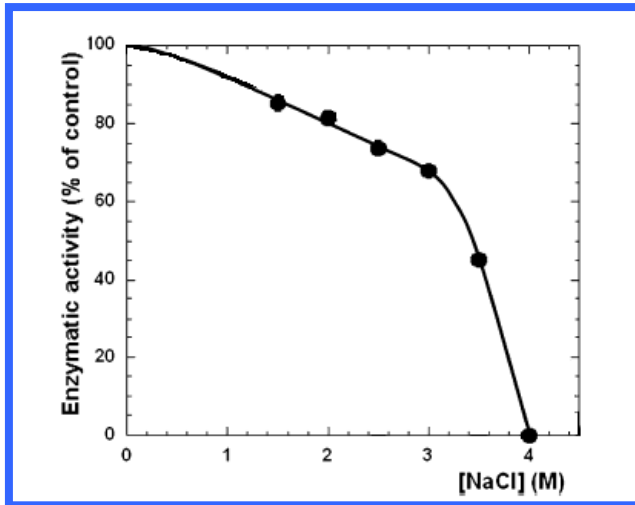
$\text{ADP} + \text{H}^+ + \text{fosfocreatina} \rightarrow \text{ATP} + \text{creatina}$



+ NaCl

Studio della struttura della creatina chinasi del muscolo (CK)

nativo dimerico



Profili di Cromatografia ad esclusione molecolare:

a: CK nativa;

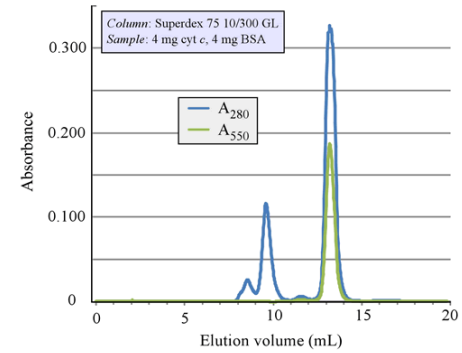
b: CK incubata in GdmHCl 0.1 M + NaCl 3.5 M;

c: CK incubata in GdmHCl 0.1 M + NaCl 3.7 M;

d: CK incubata in GdmHCl 0.1 M + NaCl 4.0 M.

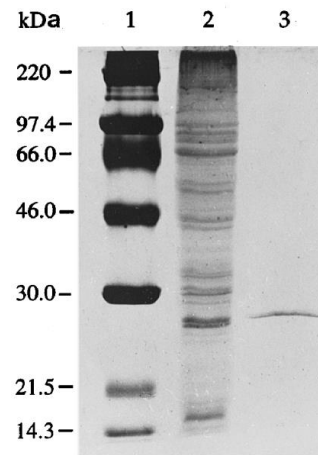
Determinazione massa molecolare; numero e peso molecolare delle subunità

✓ Cromatografia per esclusione
molecolare

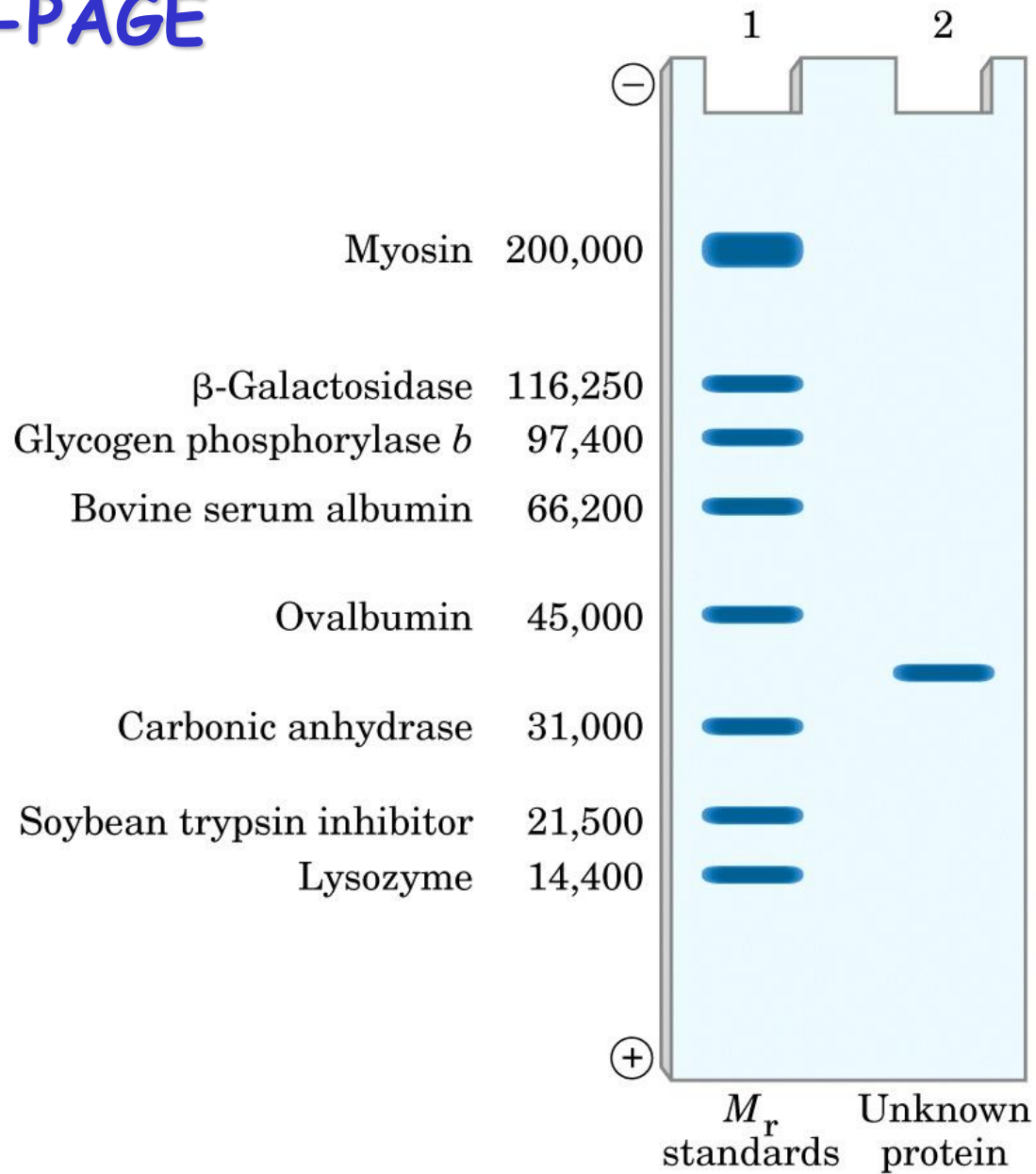


✓ SDS-PAGE

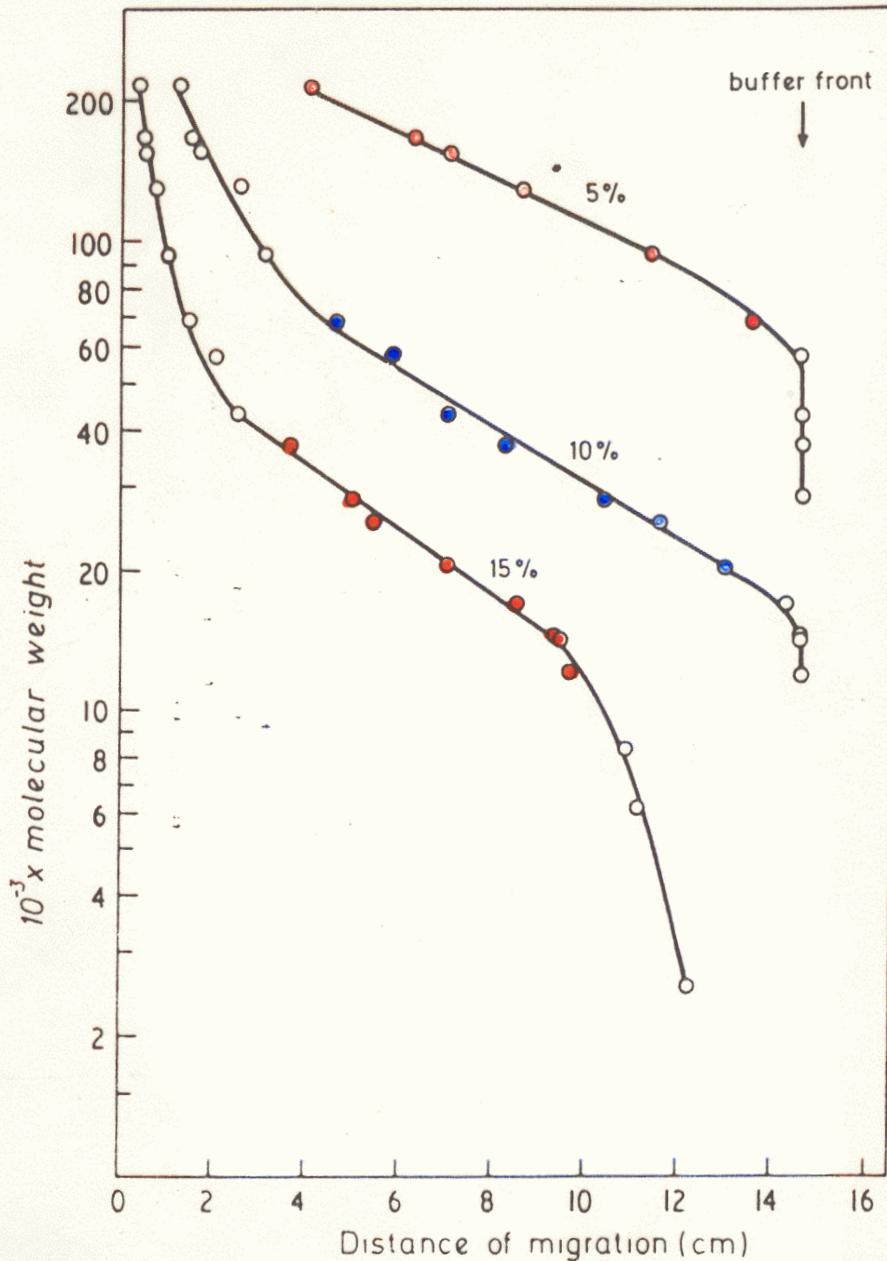
✓ Protein Cross-Linking



SDS-PAGE



Calibration curves of $\log_{10}$ polypeptide molecular weight versus distance of migration during SDS-PAGE in slab gels



The polyacrylamide gels used were uniform concentration 5%, 10% or 15%. The polypeptide markers, in order of decreasing molecular weight, were **myosin** (mol. wt. 212,000), **RNA polymerase β'** (165,000) and **β** (155,000) **subunits**, **β -galactosidase** (130,000), **phosphorilase a** (92,500), bovine serum albumin (68,000), catalase (57,500), ovalbumin (43,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), chymotrypsinogen A (25,700), soybean trypsin inhibitor (20,100), horse heart myoglobin (16,950), **horse heart myoglobin cyanogen bromide cleavage fragment I + II** (14,404), **lysozyme** (14,300), **cytochrome c** (11,700).

Characterization and deglycosylation of metalloproteinase, *Bothrops leucurus* venom (leuc-B) with PNGase

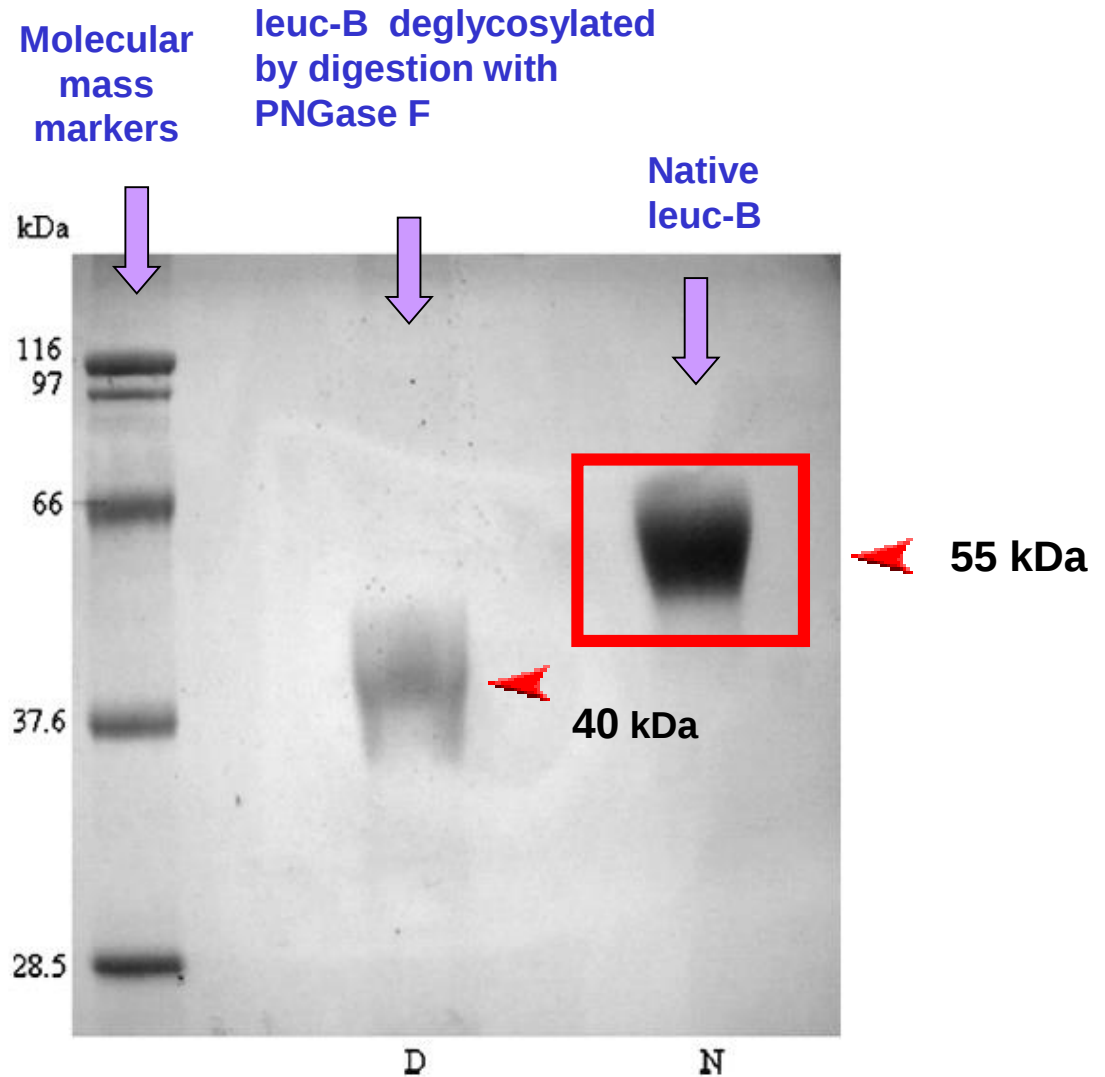


Bothrops leucurus

- Venoms produced by poisonous snakes are cocktails of biologically active proteins and peptides which kill or weaken their preys
- Leucurolysin-B (leuc-B) is an hemorrhagic metalloproteinase found in the venom of *Bothrops leucurus* (white-tailed-jararaca) snake
- PNGase is an endoglycosidase that specifically removes N-linked glycans from glycoproteins

Characterization and deglycosylation of metalloproteinase, *Bothrops leucurus* venom (leuc-B) with PNGase

**SDS-PAGE (12%,
reducing
conditions with
 β -mercaptoethanol)**



PNGase F= N-glycosidase

Fig. 3. Characterization and deglycosylation of leuc-B.

Cross-Linking delle proteine

- Il termine cross-linking indica il processo chimico con il quale due o più molecole vengono unite mediante legami covalenti.
- I reagenti di cross-linking (cross-linkers) sono molecole che contengono due o più gruppi reattivi in grado di legarsi chimicamente a specifici gruppi funzionali quali ad esempio ammine primarie, tioli, etc, presenti in molecole o proteine.
- Nel caso delle proteine, lo scopo della reazione di cross-linking è la formazione di un legame covalente tra residui spazialmente vicini in una singola catena polipeptidica, tra residui su catene peptidiche differenti o tra catene proteiche e materiali solidi.

Cross-Linking delle proteine

Tipologie di cross-linking e relative applicazioni

Tra due residui nella stessa proteina	Cross-linking intramolecolare	Stabilizza la struttura terziaria o quaternaria della proteina
Tra due residui di diverse proteine	Cross-linking intermolecolare	Stabilizza le interazioni proteina-proteina (es. complesso anticorpo-antigene)
Tra residui proteici e gruppi chimici su supporti solidi (vetro, resine)	Immobilizzazione	Consente saggi di attività o procedure di purificazione

Tipologie di prodotti comuni delle reazioni di crosslinking



cross-link

inter/intraprotein link
interpeptide link
type 2 cross-link

two connected peptides



loop-link

intrapeptide link
type 1 cross-link

single peptide



mono-link

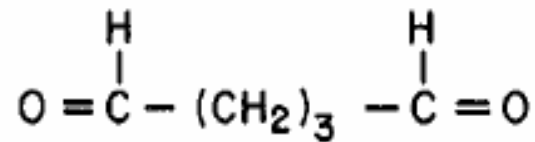
dead-end link
type 0 cross-link

single peptide

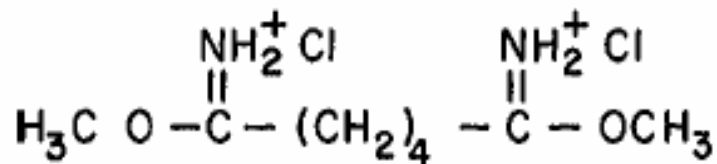
Cross-Linking delle proteine

- I cross-linkers che si usano con le proteine sono molecole che contengono due siti reattivi connessi mediante una regione detta «braccio spaziatore» tipicamente costituita da una catena alchilica
- Nella maggior parte dei casi i siti reattivi del cross-linker attaccano il gruppo amminico primario dei residui di lisina delle proteine
- Tra i reagenti più usati si possono annoverare dialdeidi ed immidi

Cross-linkers omo-bifunzionali

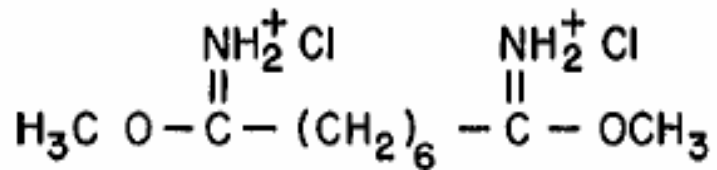


Glutaraldehyde



Dimethyl adipimidate

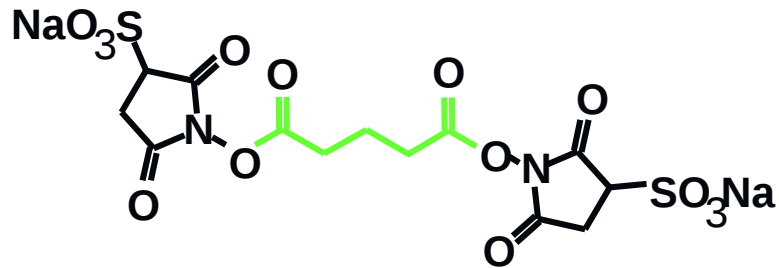
Lunghezza braccio spaziatore 8.6Å



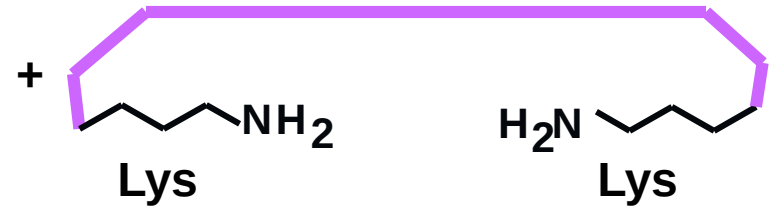
Dimethyl suberimidate

Lunghezza braccio spaziatore 11Å

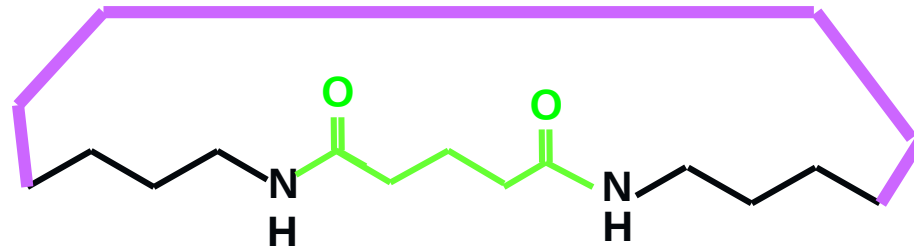
Cross-linkers omo-bifunzionali



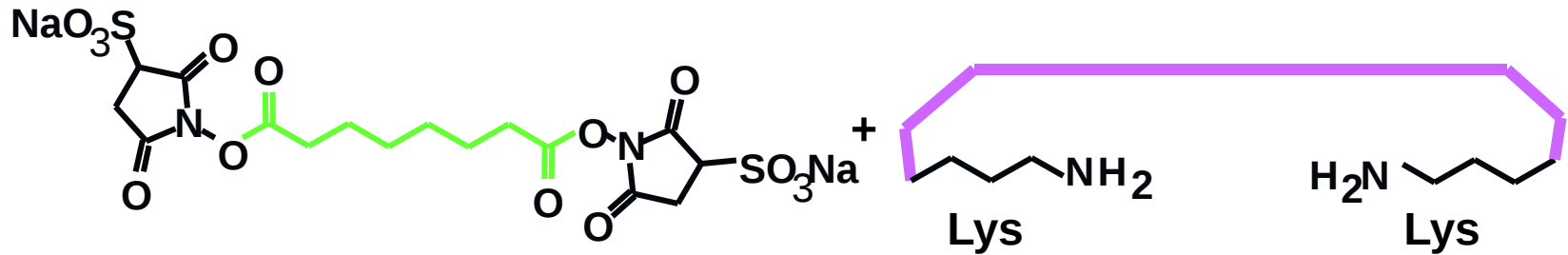
Bis(Sulfosuccinimidyl)glutarate (BS²)
lunghezza del braccio spaziatore
7.7 Å



Protein

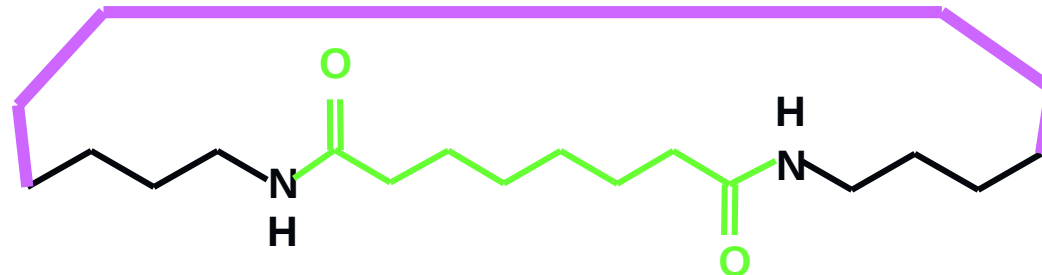


Cross-linkers omo-bifunzionali



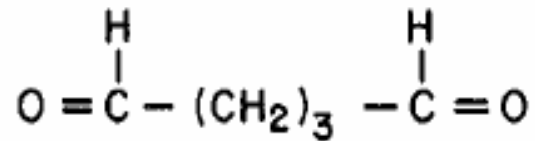
Bis(Sulfosuccinimidyl)suberate (BS³)
lunghezza del braccio spaziatore
11.4 Å

Protein

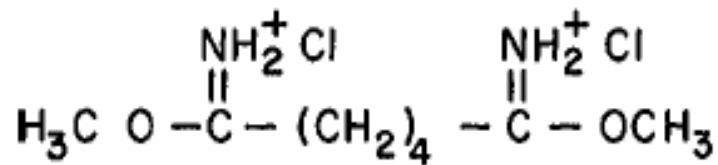


Cross-Linked Protein

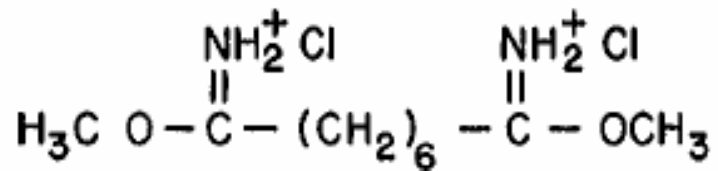
Cross-linkers omo-bifunzionali



Glutaraldehyde



Dimethyl adipimidate 8.6Å,



Dimethyl suberimidate 11Å

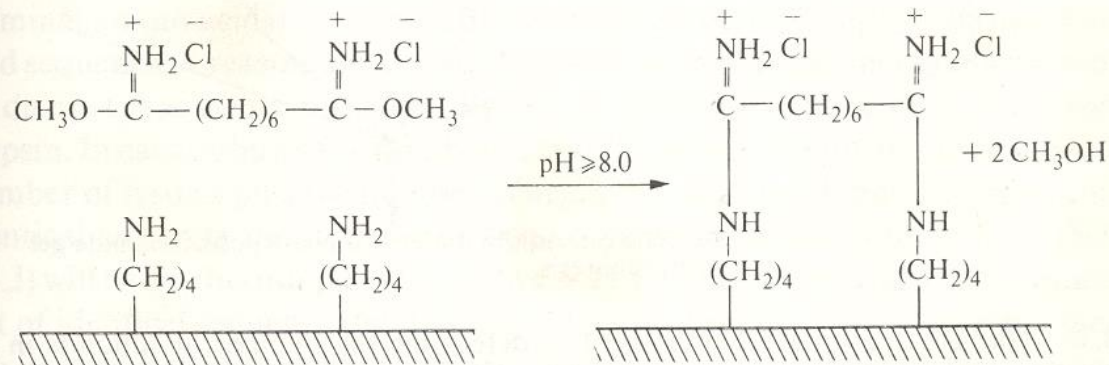


Fig. 3.35. Cross-linking of lysine side chains by reaction with dimethylsuberimide.

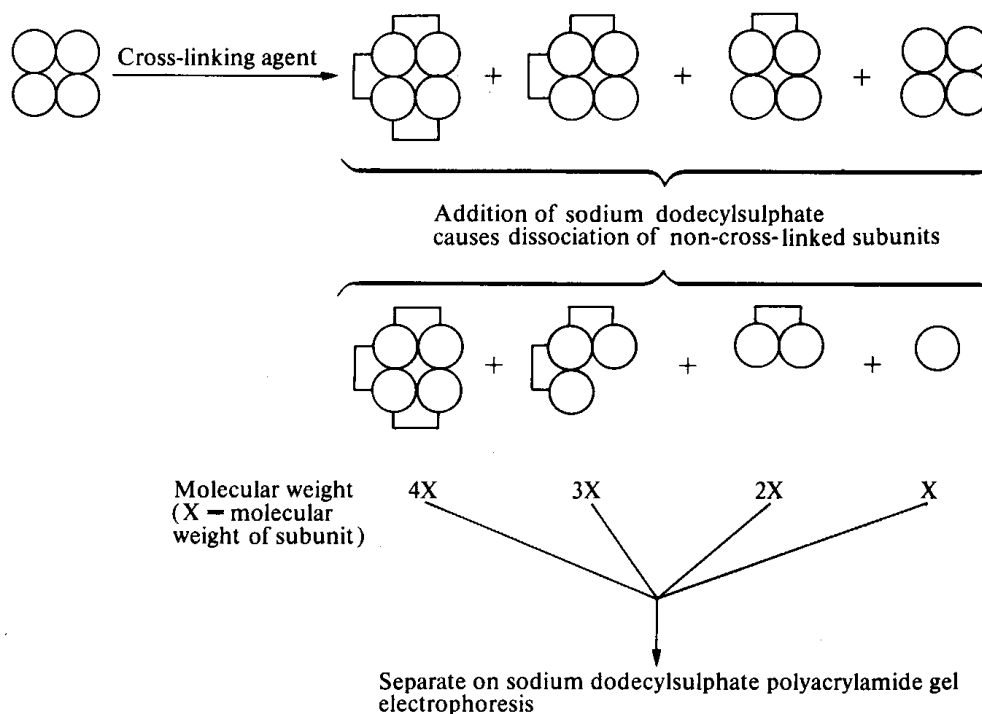


Fig. 3.36. The use of a cross-linking agent to determine the number of subunits in an oligomeric enzyme. For convenience the intrasubunit cross-linkages are not shown.

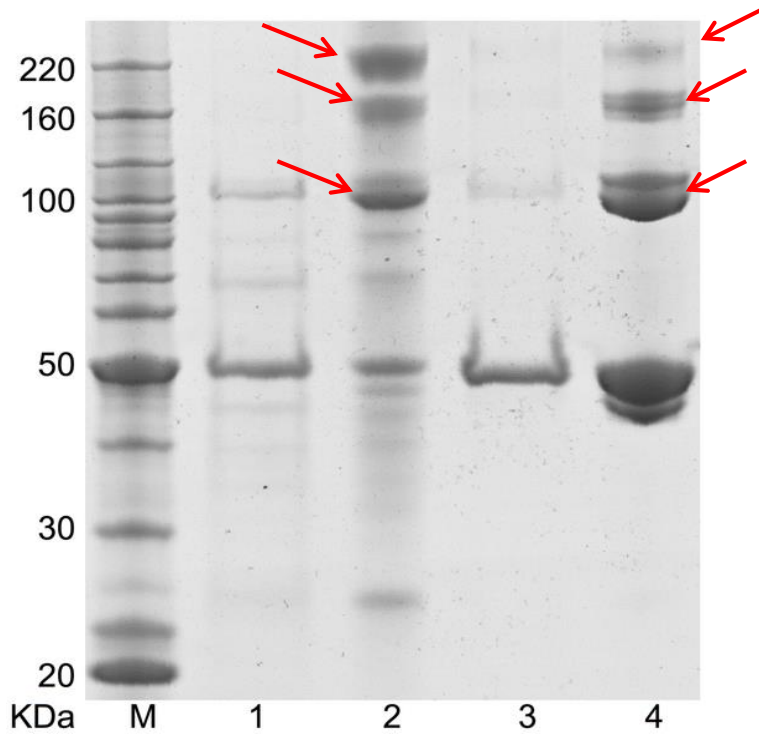
A Novel *N*-Acetylglutamate Synthase Architecture Revealed by the Crystal Structure of the Bifunctional Enzyme from *Maricaulis maris*

Dashuang Shi^{1*}, Yongdong Li^{1,2}, Juan Cabrera-Luque¹, Zhongmin Jin³, Xiaolin Yu¹, Gengxiang Zhao¹, Nantaporn Haskins¹, Norma M. Allewell⁴, Mendel Tuchman¹

N-acetylglutamate synthase/kinase (NAGS/K) that catalyze the first two steps of arginine biosynthesis and is homologous to vertebrate *N*-acetylglutamate synthase (NAGS), an essential cofactor-producing enzyme in the urea cycle, was identified in *Maricaulis maris* (mmNAGS/K)

The quaternary structure of crystalline NAGS/K from *Xanthomonas campestris* (xcNAGS/K) is similar, and cross-linking experiments indicate that both mmNAGS/K and xcNAGS are tetramers in solution.

SDS-PAGE: Cross-linking of recombinant xcNAGS/K and mmNAGS/K proteins with dimethyl suberimidate.



Lanes

M, protein size markers;

1, xcNAGS/K without cross-linking reagent;

2, xcNAGS/K with cross-linking reagent;

3, mmNAGS/K without cross-linking reagent;

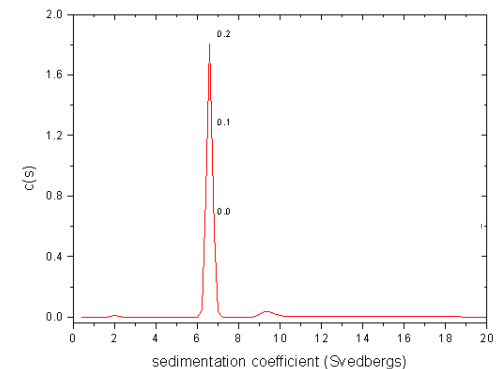
4, mmNAGS/K with cross-linking reagent.

mmNAGS/K and xcNAGS/K (0.15 μ g) were incubated with **dimethyl suberimidate** (0.25 μ g) in 50 μ l solution containing 200 mM triethanolamine, pH 8.25 for three hours at 298 K.

To determine the state of oligomerization of both xcNAGS/K and mmNAGS/K in solution, cross-linking experiments were performed with dimethyl suberimidate as the cross-linking agent. Four major bands were seen for both enzymes with SDS-PAGE, with molecular weights corresponding to oligomers of 1, 2, 3 and 4 subunits (Lane 2 and 4). Thus, xcNAGS/K and mmNAGS/K appear to exist primarily as tetramers in solution.

Determinazione massa molecolare; numero e peso molecolare delle subunità

✓ Ultracentrifugazione analitica

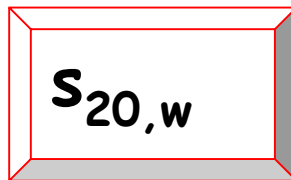


$$v = \frac{dr}{dt} = \frac{2}{9} \frac{r_p^2 (d_p - d_m)}{\eta} \omega^2 r$$

$$v = s \omega^2 r$$

s = coefficiente di sedimentazione =
velocità di sedimentazione / unità di campo centrifugo

$$s = \frac{v}{\omega^2 r} \quad s = \frac{dr}{dt} \frac{1}{\omega^2 r}$$



unità **S**vedberg (S) = 10^{-13} s

La velocità di sedimentazione di una particella può essere espressa in termini di coefficiente di sedimentazione (s) che corrisponde alla velocità di sedimentazione per unità di campo centrifugo applicato. Il coefficiente di sedimentazione ha le dimensioni di un tempo e si misura in *Svedberg, S*.

$$1 S = 10^{-13} \text{ sec}$$

Esempio: $s = 5 \times 10^{-13} \text{ sec}$ corrisponde a 5S

<u>Specie sedimentate</u>	<u>Coeff. di sedimentazione</u>
Enzimi, ormoni e proteine	2 - 25S
Acidi nucleici	3 - 100S
Ribosomi, polisomi	20 - 200S
Virus	40 - 1000S
Lisosomi	4000S
Membrane	100 - 100000S
Mitocondri	20000 - 70000S

Coefficiente di sedimentazione di alcune particelle biologiche

GRUPPO	PARTICELLA	$S_{20,W}$ (Svedberg)
Proteine	Citocromo c	1,7
	Mioglobina	1,82
	Insulina	1,95
	Tripsina	2,5
	Pepsina	2,8
	Collageno	4,0
	Emoglobina	4,1
	Albumina (plasma)	4,5
	Miosina	64
Acidi nucleici	RNA - Transfer	4
	RNA - ribosomale (procarioti)	5
		16
		23
	RNA - ribosomale (eucarioti)	5
		5,8
		18 -25/28

Virus	Virus della poliomelite	154
	Virus del mosaico del tabacco (TMV)	180
	SV 40	240
	Virus dell'influenza	700
Organelli cellulari	Subunità ribosomali (procarioti)	30 (piccola)
		50 (grande)
	Subunità ribosomali (eucarioti)	40
		60
	Ribosomi (monomeri) (procarioti)	70
	Ribosomi (monomeri) (eucarioti)	80
	Frammenti di membrane	da 10^2 a 10^4
	Membrane plasmatiche	$> 10^5$
	Reticolo endoplasmatico liscio /rugoso	10^3
	Lisosomi	da $4 \cdot 10^3$ a $2 \cdot 10^4$
	Perossisomi	$4 \cdot 10^3$
	Mitocondri	da $1 \cdot 10^4$ a $7 \cdot 10^4$
	Cloroplasti	da 10^5 a 10^6
	Nuclei	da 10^6 a 10^7
	Cellule intere	da 10^7 a 10^8

Determinazione della massa molecolare relativa

Velocità di sedimentazione

$$s = \frac{dr}{dt} \frac{1}{\omega^2 r} \quad s \omega^2 dt = \frac{dr}{r} \quad \text{integrando} \quad s \omega^2 t = \ln r$$

$$M_r = \frac{RTs}{D(1 - \bar{v} \rho)}$$

R = costante universale dei gas

T = temperatura assoluta

D = coefficiente di diffusione

$\bar{v}$ = volume parziale specifico del soluto

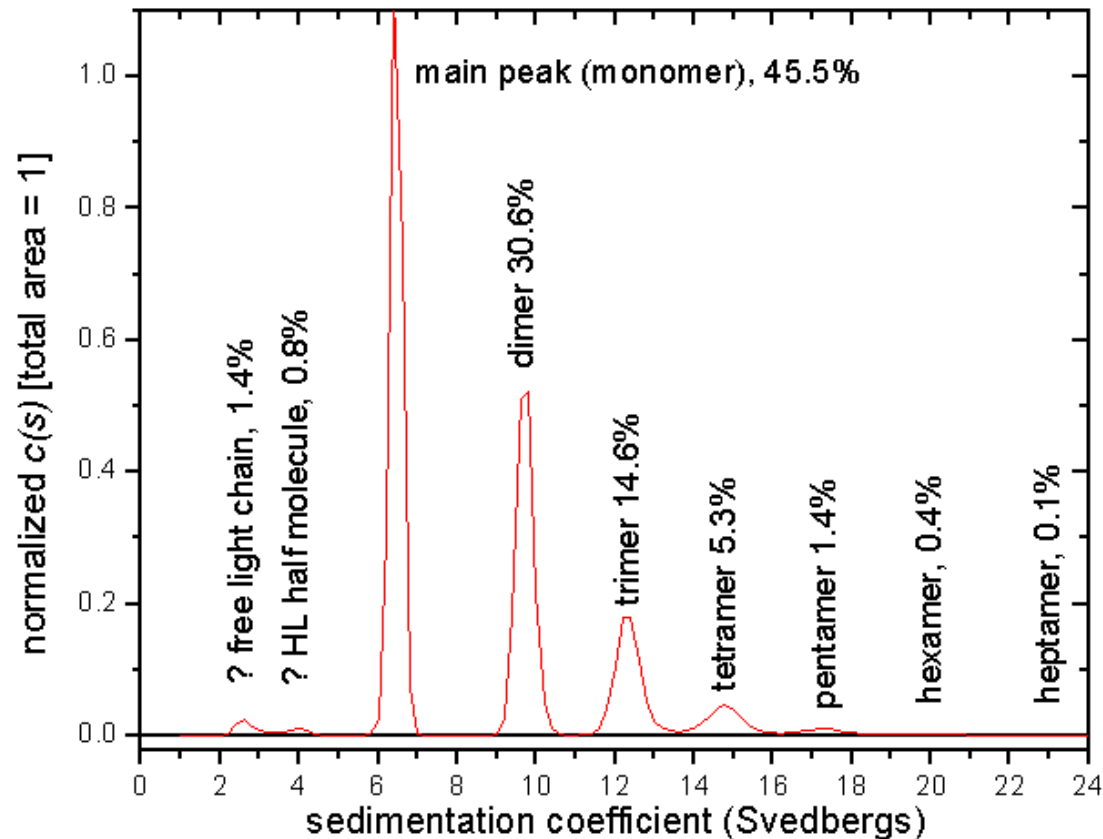
ρ = densità del solvente

Equilibrio di sedimentazione

$$M_r = \frac{2 R T \ln(c_2/c_1)}{\omega^2 (1 - \bar{v} \rho) (r_2^2 - r_1^2)}$$

c_1 = concentrazione misurata a distanza r_1

c_2 = concentrazione misurata a distanza r_2



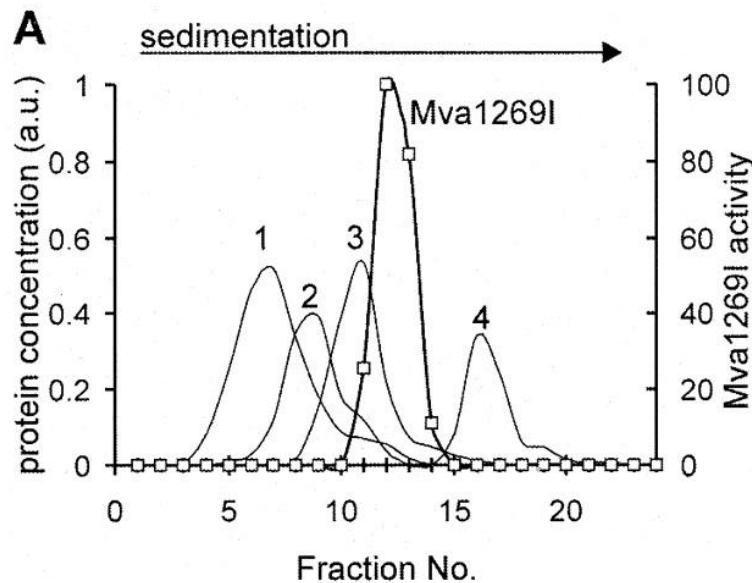
The figure shows results for a highly stressed sample of a monoclonal antibody. We see well-resolved peaks for dimer, trimer, ... out to heptamer, as well as 2 peaks for fragments. (The hexamer and heptamer peaks are too small to see on this scale.) This resolution is much better than can be achieved by size-exclusion chromatography

Mva1269I: A Monomeric Type IIS Restriction Endonuclease from *Micrococcus Varians* with Two EcoRI- and FokI-like Catalytic Domains*

Received for publication, June 22, 2005, and in revised form, September 20, 2005 Published, JBC Papers in Press, October 11, 2005, DOI 10.1074/jbc.M506775200

Elena Armalyte^{‡1}, Janusz M. Bujnicki^{§2}, Jolanta Giedriene[‡], Giedrius Gasiunas[‡], Jan Kosiński^{§3}, and Arvydas Lubys^{‡4}

Ultracentrifugation analysis of wt Mva1269



A, analysis of ultracentrifugation fractions of wt Mva1269I.

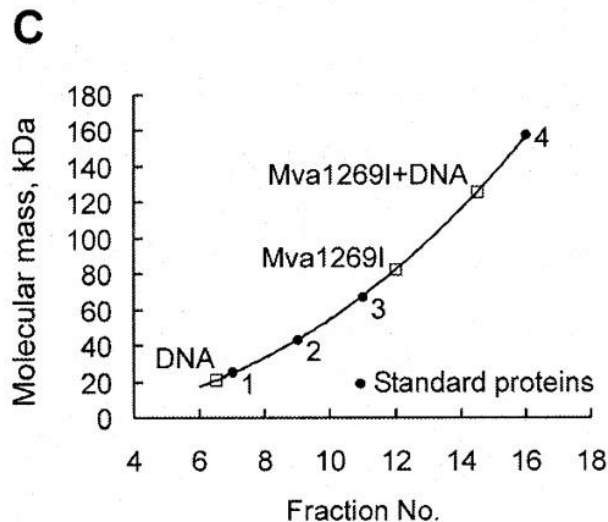
marker proteins:

1, chymotrypsinogen (25 kDa);

2, ovalbumin (43 kDa);

3, bovine serum albumin (66 kDa);

4, aldolase (158 kDa).



B, the standard protein curve, derived from the peak positions of the marker proteins (numbering of standards is the same as in A).

To locate Mva1269I peaks, fractions were tested for Λ DNA cleavage activity

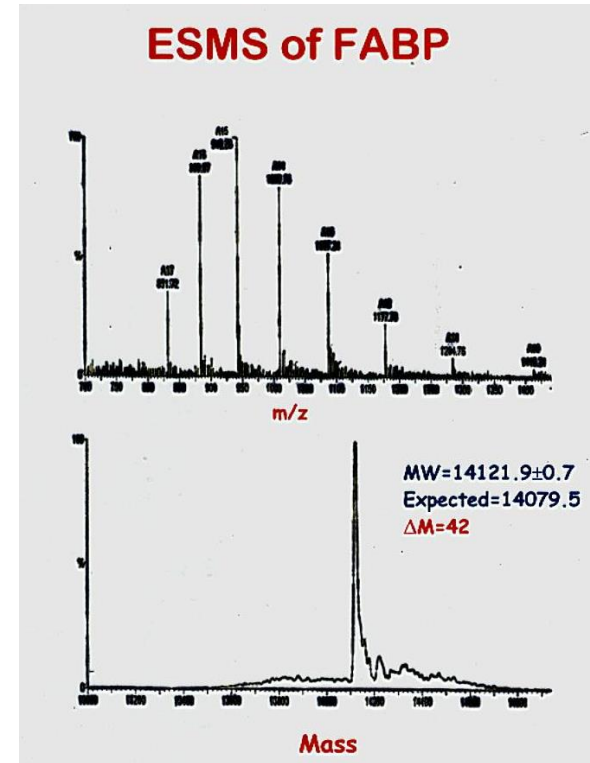
Mva1269I Is a Monomer in Solution—The quaternary structure of free and DNA-bound Mva1269I was examined by **sucrose gradient velocity** centrifugation. Centrifugation of Mva1269I in the absence of specific DNA showed that Mva1269I sediments as a protein of **82,000**. The calculated M_r of Mva1269I is **78,400**; results, therefore, suggest that Mva1269I is a **monomer** in the absence of specific DNA.

Mva1269I Monomer Forms a Complex with DNA Molecule

To study Mva1269I oligomerization in the presence of specific DNA, the enzyme was mixed either with radioactively labeled specific DNA oligoduplex and subjected to sucrose gradient centrifugation. After centrifugation, individual fractions were tested for Mva1269I activity and for their radioactivity. Two peaks of radioactivity were observed. One of them represented the free DNA that, relative to the marker proteins, yielded an apparent molecular weight of **21,000** (the calculated M_r is **14,600**). The increased estimated molecular weight of free DNA can be explained by its cylindrical shape, resulting in increased hydrodynamic friction compared with that experienced by spherically shaped marker proteins. The second radioactivity peak overlapped with the peak of Mva1269I activity, suggesting that Mva1269I and specific DNA form a complex with apparent M_r of **124,000** ($82,000 + 21,000 \times 2 = 124,000$).

Determinazione massa molecolare; numero e peso molecolare delle subunità

✓ Spettrometria di massa



✓ Light scattering

