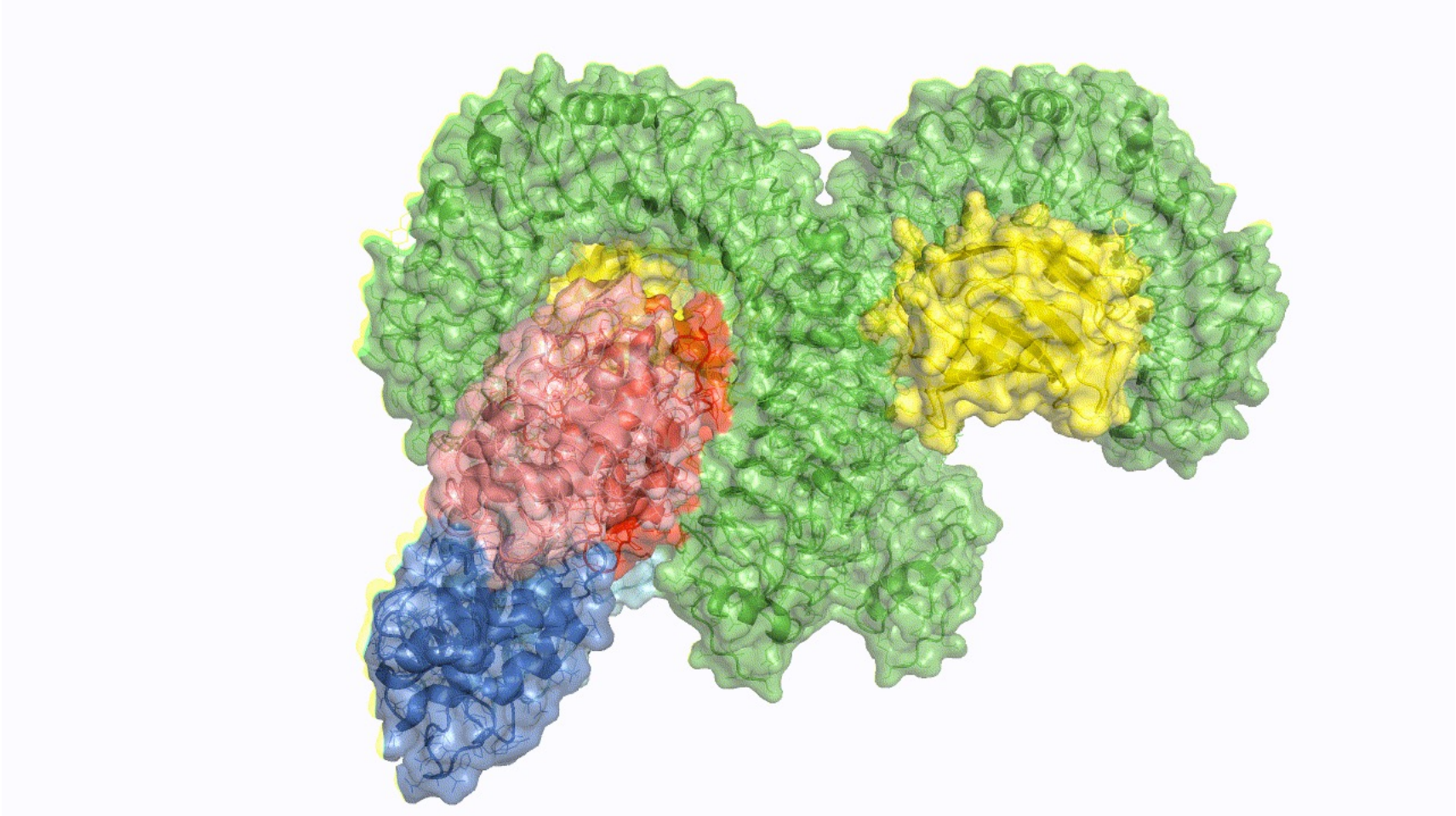


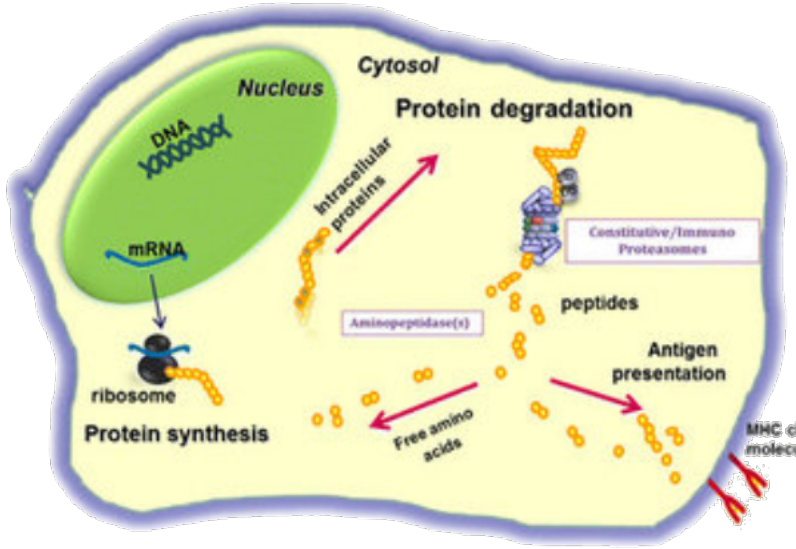
Proteins as Intelligent Polymers: Large Structural/Functional Changes upon small Physical or Chemical specific stimuli

Mauro Angeletti
Massimiliano Cuccioloni
Anna Maria Eleuteri

TOPICS

- Proteasome: a large multi-enzymatic system
  - Proteasome: roles
  - Proteasome: structure
  - Proteasome: enzymatic functions
  - Proteasome: inhibitors and effectors
  - *Strategies: enzymatic assays, fluorescent assays, western-blots, cell cultures assays*
- HMG-CoA reductase
  - Role
  - Control by EGCG
  - *Strategies: HPLC enzymatic assays, kinetics on SPR e Q-balance biosensors, virtual screening*
- Ovalbumin: a temperature proteic sensor
  - Structure
  - Function
  - *Strategies: enzymatic assays, DSC*

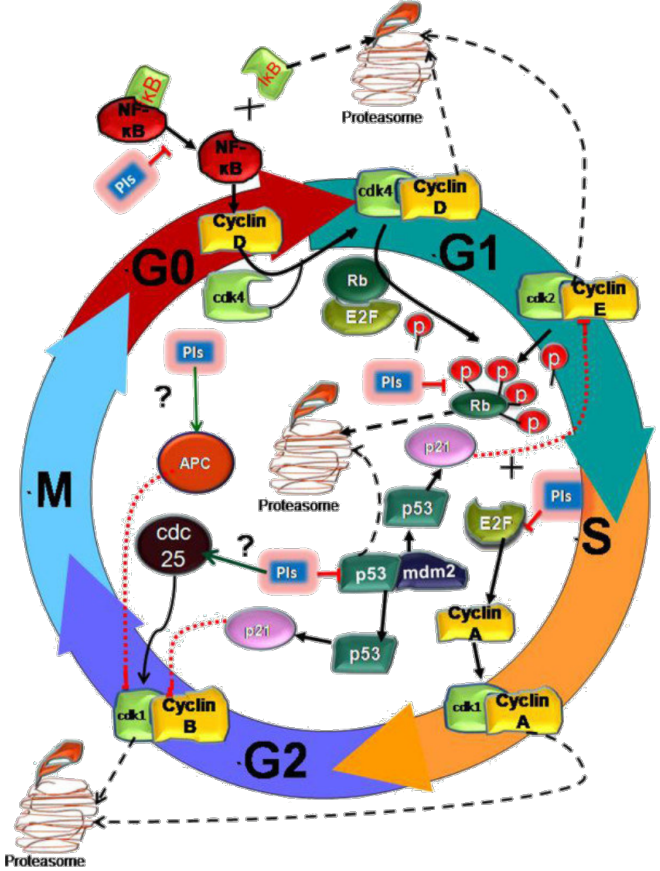
1. Proteasome: Major Roles

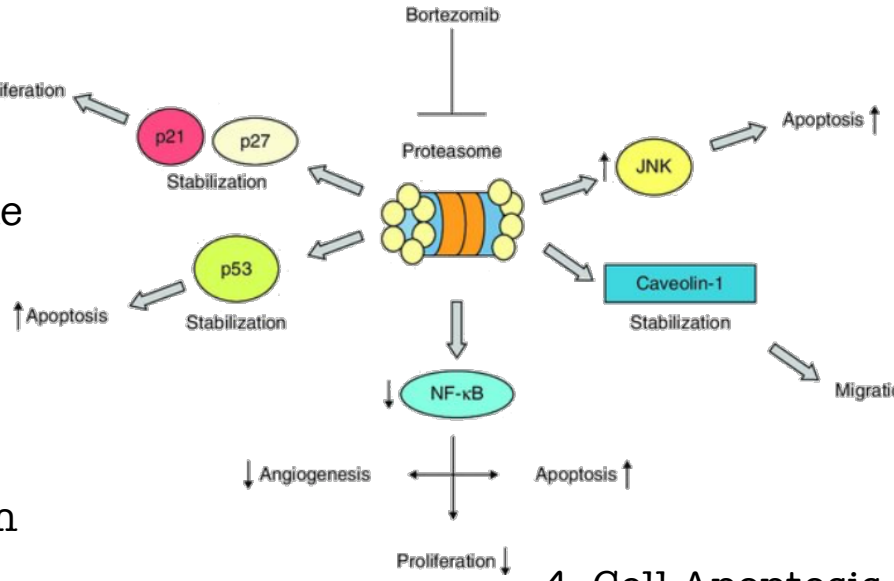


1. The proteasome is the key-enzyme in protein turnover/recycling

2. In addition, the proteasome-generated peptides and their presentation to the MHC class I, are part of the cellular immunity system

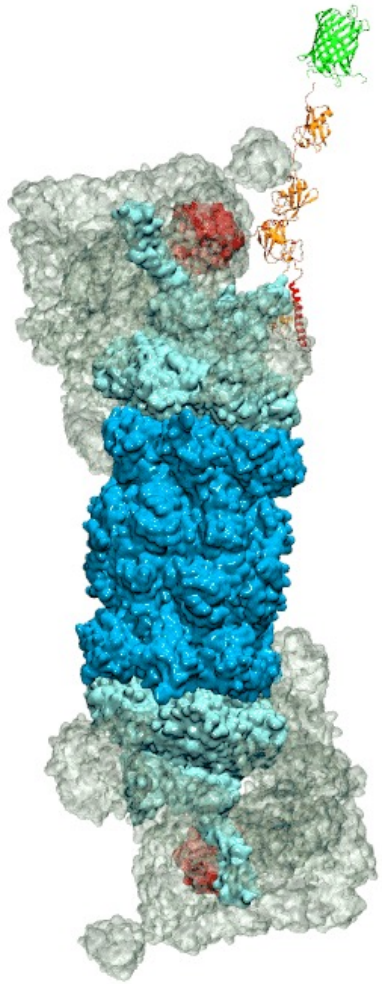
3. Cell cycle phases are finely regulated by several proteins, which are proteasome substrates



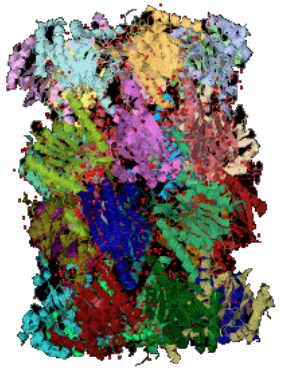


4. Cell Apoptosis, Migration and Proliferation are controlled by specific proteins whose levels are controlled byproteasome

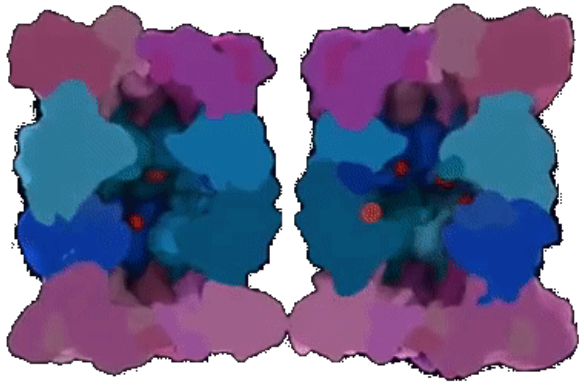
1. Proteasome: Structure



1. Complex quaternary structure:
   - 26S particle
   - catalytic 20S
   - ATP-unfoldase caps (lid/base) 19S

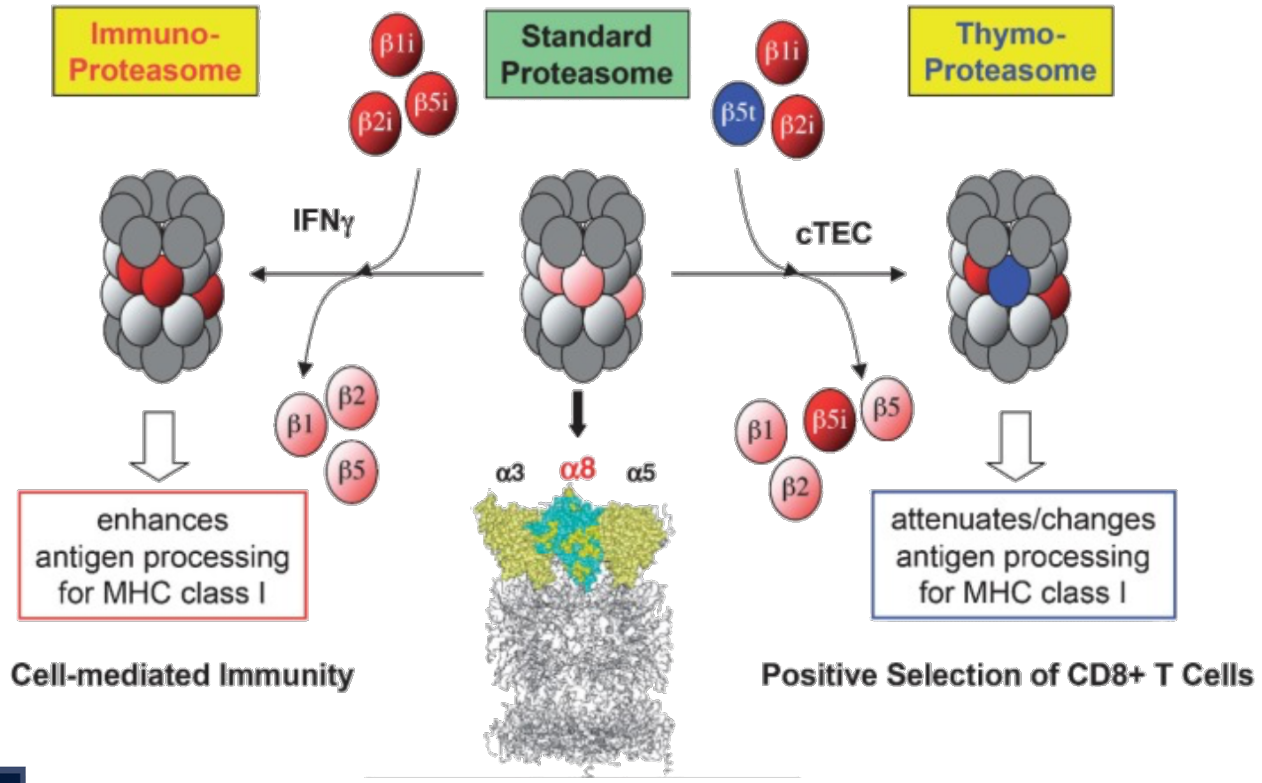


2. The 20S proteasome is composed by 4 stacked-rings (each ring is 7 subunits). MW ≈ 700 KDa



3. The central rings form a cavity (remember that proteasome substrates are usually ***unfolded*** proteins)

1. Proteasome: structure/subunits composition



1. Under specific conditions, the proteasome subunits composition can be changed.

2. The reaulting complexes have different cell functions

cortical thymic epithelial cells (cTECs)

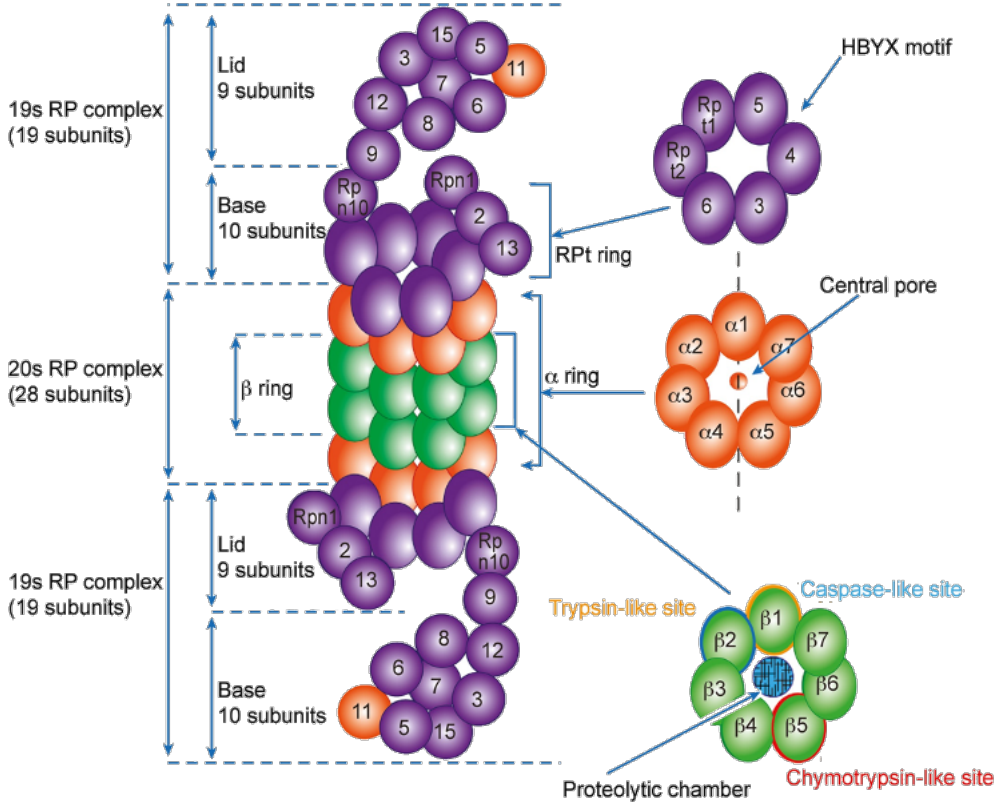
1. Proteasome: Enzymatic functions

The proteasome has several specific proteolytic functions:

- Trypsin-like activity (subunits $ß_2$)
- Chymotrypsin-like activity ($ß_5$)
- Caspase-like activity ($ß_1$)

The central rings contain the catalytic sites.

Threonine 1 is the catalytic residue for each catalytic subunit, able to act as a nucleophile during the peptide bond hydrolysis (similarly to serine proteases).

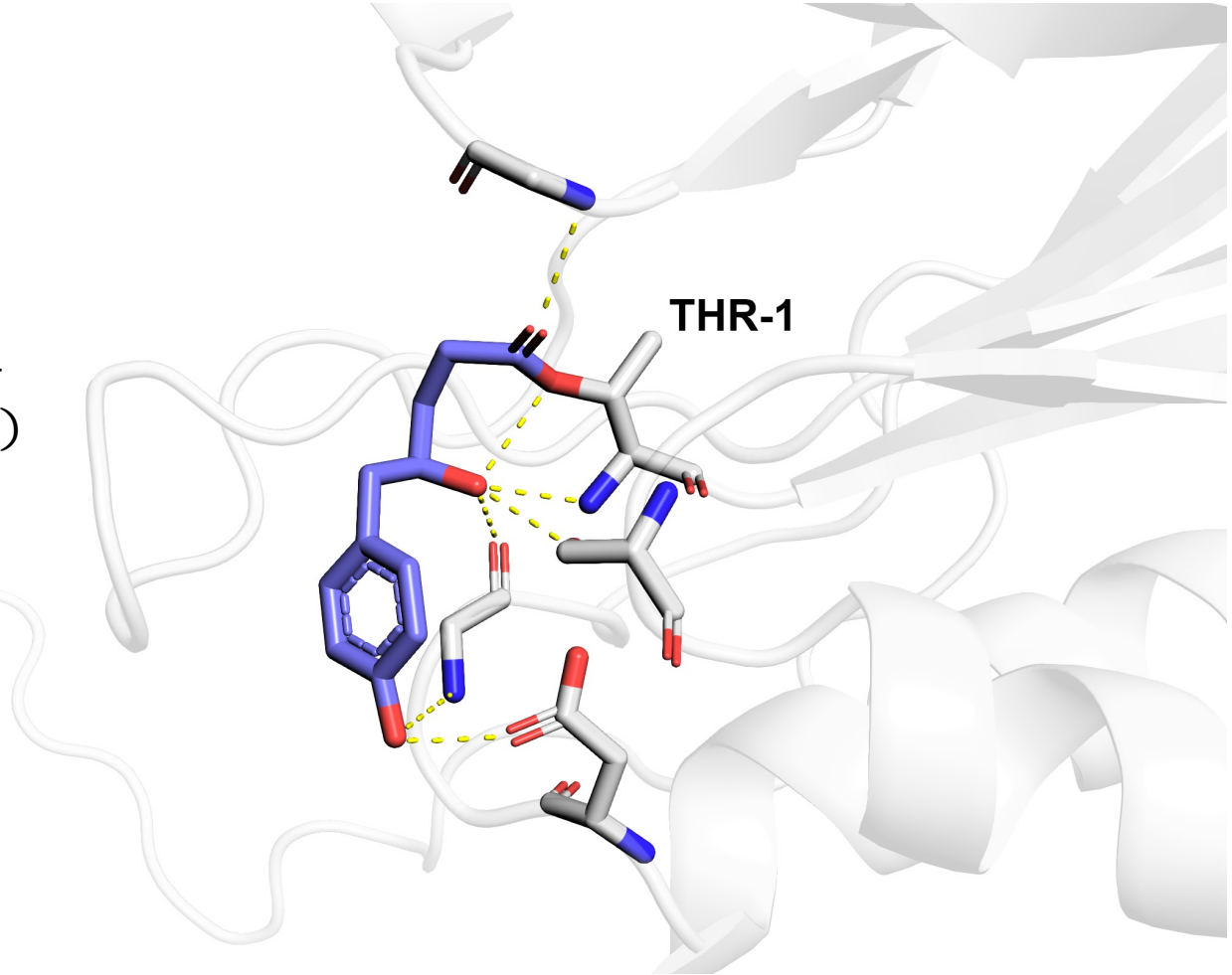


1. Proteasome: Inhibitors and Effectors

A non-esaustive list of the 20S Proteasome modulators:

Reversibly:
Small ions as Na^+
Several plant metabolites (epigallocatechin-3-gallate, quercetin, luteolin, gallic acid, etc) with Kd= 10^{-4}-10^{-5} M)
Semi-Synthetic ligands (i.e. Arene-Ru(II) complexes of curcumin (Pettinari's Group))
The modulators have different affinities for the $ß_1$, $ß_2$, $ß_5$ and $ß_{1i}$, $ß_{2i}$, $ß_{5i}$ subunits

Irreversibly:
Phenyl-γ-valerolactones (polyphenols microbial metabolites)



Strategies:

- *enzymatic assays*
- *fluorescent assays*
- *western-blots*
- *cell cultures assays*
- *Docking*

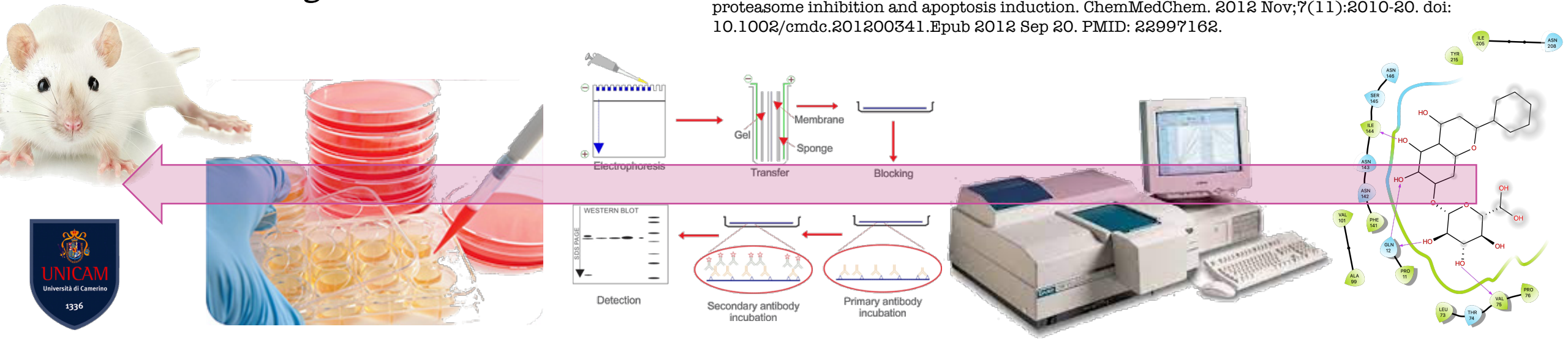
1: Cuccioloni M, Bonfili L, Mozzicafreddo M, Cecarini V, Scuri S, Cocchioni M, Nabissi M, Santoni G, Eleuteri AM, Angeletti M. Mangiferin blocks proliferation and induces apoptosis of breast cancer cells via suppression of the mevalonate pathway and by proteasome inhibition.
Food Funct. 2016 Oct 12;7(10):4299-4309. doi: 10.1039/c6fo01037g. PMID: 27722367.

2: Cecarini V, Bonfili L, Cuccioloni M, Mozzicafreddo M, Angeletti M, Keller JN, Eleuteri AM. The fine-tuning of proteolytic pathways in Alzheimer's disease.
Cell Mol Life Sci. 2016 Sep;73(18):3433-51. doi: 10.1007/s00018-016-2238-6. Epub 2016 Apr 27. PMID: 27120560.

3: Cecarini V, Bonfili L, Cuccioloni M, Mozzicafreddo M, Rossi G, Keller JN, Angeletti ,M, Eleuteri AM. Wild type and mutant amyloid precursor proteins influence downstream effects of proteasome and autophagy inhibition. Biochim Biophys Acta. 2014 Feb;1842(2):127-34. doi: 10.1016/j.bbadis.2013.11.002. Epub 2013 Nov 8. PMID: 24215712.

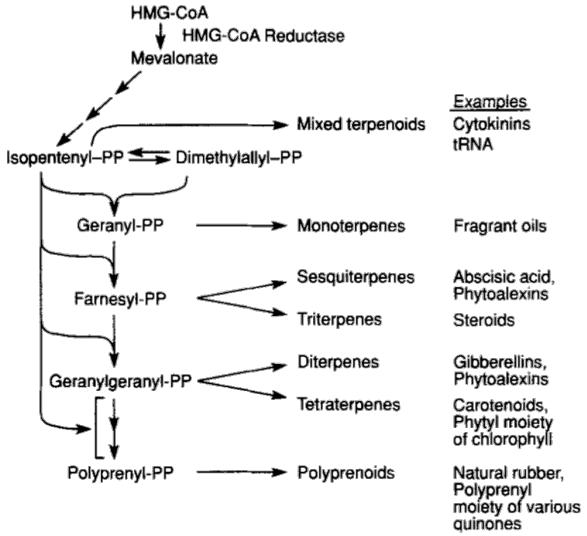
4: Bonfili L, Cuccioloni M, Cecarini V, Mozzicafreddo M, Palermo FA, Cocci P, Angeletti ,M, Eleuteri AM. Ghrelin induces apoptosis in colon adenocarcinoma cells via proteasome inhibition and autophagy induction. Apoptosis. 2013 Oct;18(10):1188-200. doi: 10.1007/s10495-013-0856-0. PMID: 23632965.

5: Bonfili L, Pettinari R, Cuccioloni M, Cecarini V, Mozzicafreddo M, Angeletti, M, Lupidi G, Marchetti F, Pettinari C, Eleuteri AM. Arene-Ru(II) complexes of curcumin exert antitumor activity via proteasome inhibition and apoptosis induction. ChemMedChem. 2012 Nov;7(11):2010-20. doi: 10.1002/cmdc.201200341.Epub 2012 Sep 20. PMID: 22997162.

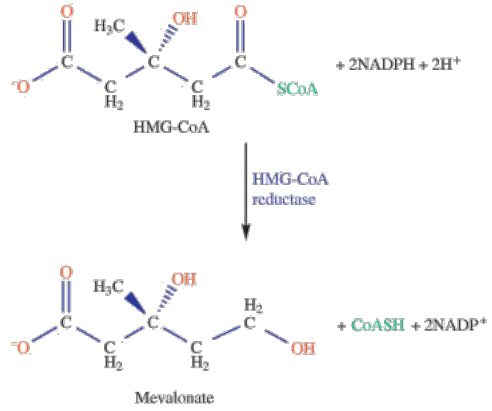


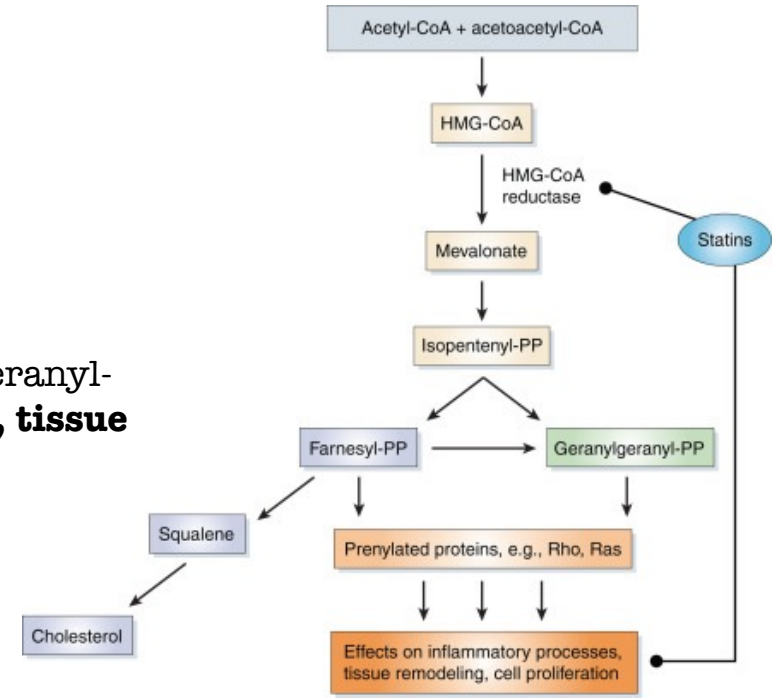
2. HMG-CoA reductase

3. In Plants, HMG-CoA reductase (HMGR)is the key enzyme of the terpenes synthesis.

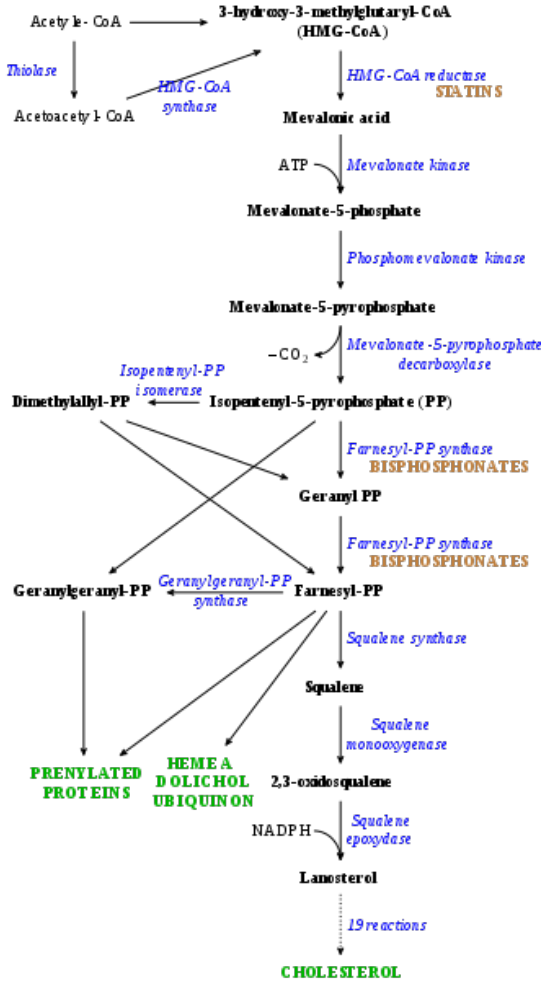


2. HMGR late products are farnesyl- and geranylgeranyl-by-products, involved in **inflammatory processes, tissue remodeling** and **cell proliferation**





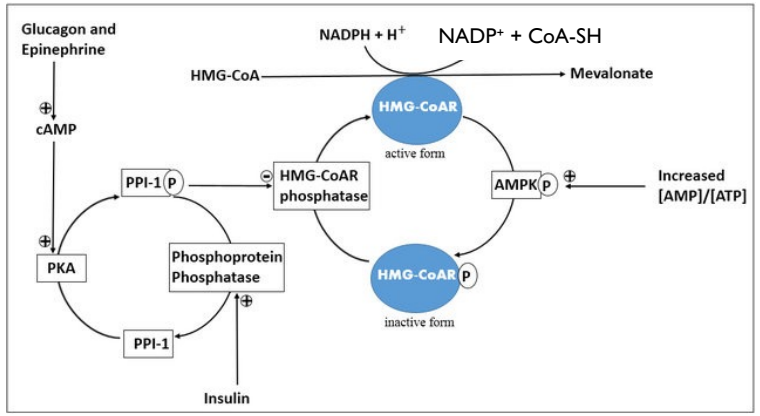
1. HMG-CoA reductase (HMGR)is the key enzyme of the cholesterol synthesis.

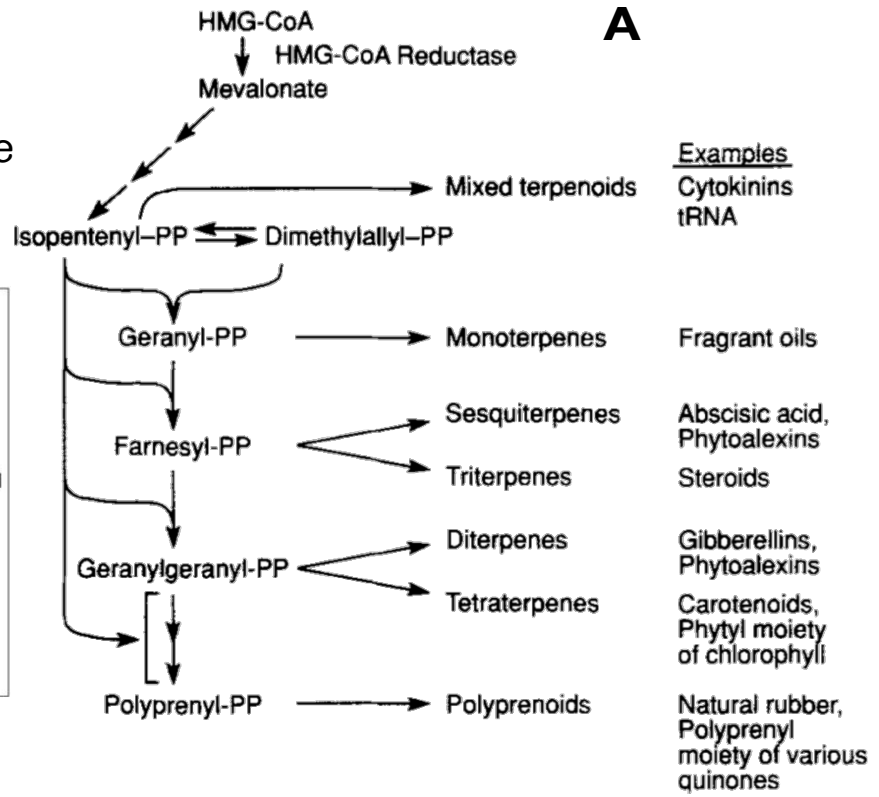


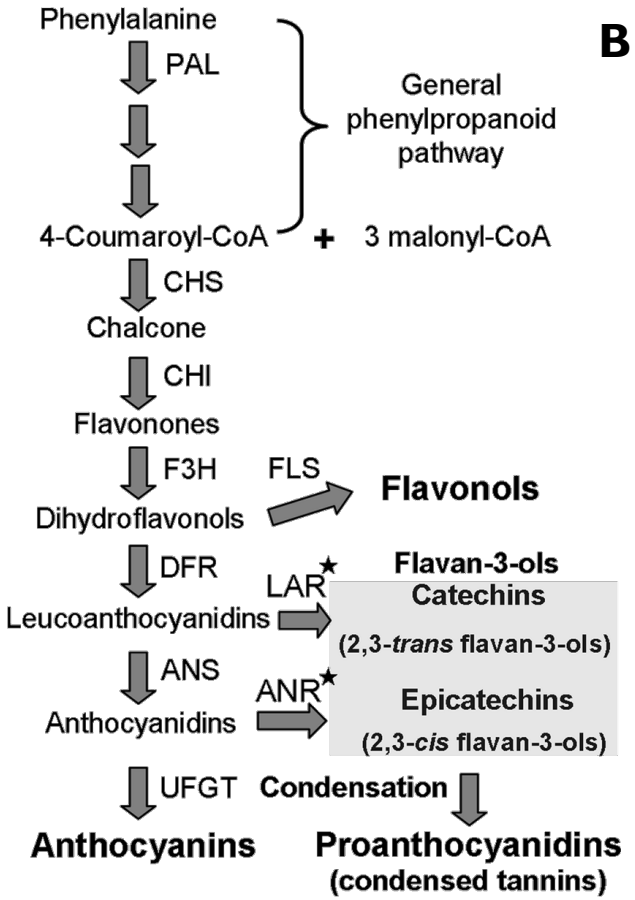
2. HMG-CoA reductase control

2. Two main pathways lead to the biosynthesis of antiparasitic/antimicrobial plant metabolites:
A. Mevalonate pathway
B. Flavonoid biosynthesis pathway

1. As any enzyme catalizing irreversible reactions, HMGR is finely controlled (see below just one example)





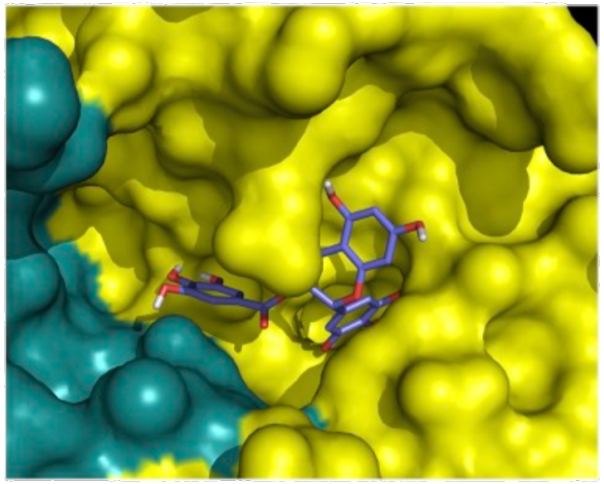


Cross-Talk Hypothesis: some metabolite of the flavonoid biosynthesis pathway could control key-enzymes of the mevalonate pathway

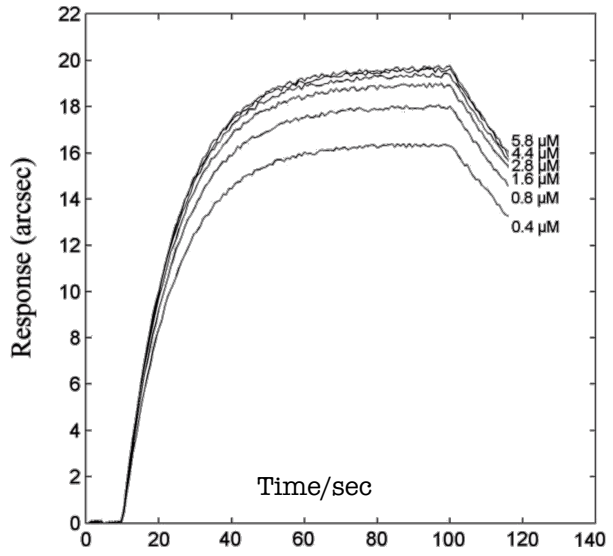
2. HMGR ligands VIRTUAL SCREENING

>1000 Metabolites of the flavonoid biosynthetic pathway (and their further by-products) have been docked to HMGR using virtual screening.

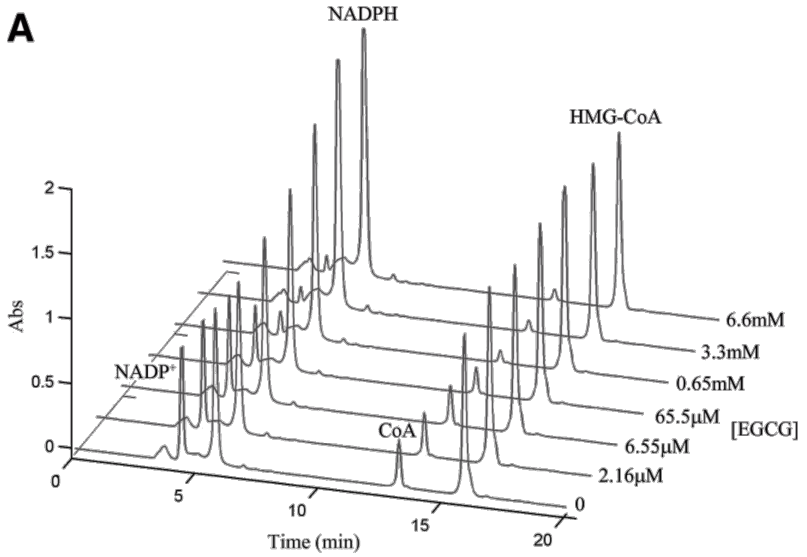
Among them, EGCG potently binds the NADP+ binding site, inhibiting HMGR.



EGCG inside the NADPH binding site (docking)



EGCG added to surface-tethered HMG-CoA reductase (SPR biosensor kinetics)



Effect of EGCG HMG-CoA reductase enzymatic activity (HPLC)

Strategies:

- *Docking and virtual screening*
- *enzymatic assays on HPLC*
- *fluorescent assays*
- *kinetics on SPR e QCM biosensors*

1: Cuccioloni M, Bonfili L, Mozzicafreddo M, Cecarini V, Scuri S, Cocchioni M, Nabissi M, Santoni G, Eleuteri AM, Angeletti M. Mangiferin blocks proliferation and induces apoptosis of breast cancer cells via suppression of the mevalonate pathway and by proteasome inhibition. Food Funct. 2016 Oct 12;7(10):4299-4309. doi: 10.1039/c6fo01037g. PMID: 27722367.

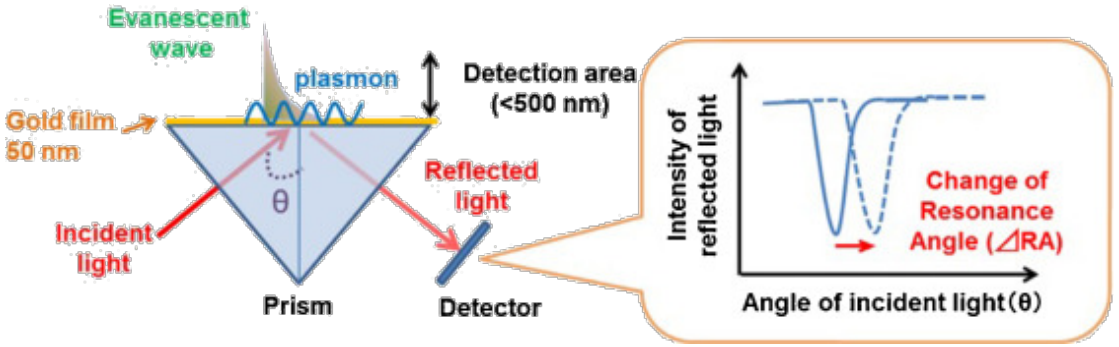
2: Palermo FA, Cocci P, Mozzicafreddo M, Arukwe A, Angeletti M, Aretusi G, Mosconi G. Tri-m-cresyl phosphate and PPAR/LXR interactions in seabream hepatocytes: revealed by computational modeling (docking) and transcriptional regulation of signaling pathways. Toxicol Res (Camb). 2015 Dec 18;5(2):471-481. doi: 10.1039/c5tx00314h. PMID: 30090361; PMCID: PMC6061042.

3: Mozzicafreddo M, Cuccioloni M, Bonfili L, Cecarini V, Palermo FA, Cocci P, Mosconi G, Capone A, Ricci I, Eleuteri AM, Angeletti M. Environmental pollutants directly affect the liver X receptor alpha activity: Kinetic and thermodynamic characterization of binding. J Steroid Biochem Mol Biol. 2015 Aug;152:1-7. doi: 10.1016/j.jsbmb.2015.04.011. Epub 2015 Apr 11. PMID: 25869557.

4: Palermo FA, Cocci P, Angeletti M, Felici A, Polzonetti-Magni AM, Mosconi G. Dietary Aloe vera components' effects on cholesterol lowering and estrogenic responses in juvenile goldfish, Carassius auratus. Fish Physiol Biochem. 2013 Aug;39(4):851-61. doi: 10.1007/s10695-012-9745-7. Epub 2012 Nov 8. PMID: 23135154.

5: Cuccioloni M, Mozzicafreddo M, Spina M, Tran CN, Falconi M, Eleuteri AM, Angeletti M. Epigallocatechin-3-gallate potently inhibits the in vitro activity of hydroxy-3-methyl-glutaryl-CoA reductase. J Lipid Res. 2011 May;52(5):897-907. doi: 10.1194/jlr.M011817. Epub 2011 Feb 25. PMID: 21357570; PMCID: PMC3073461.

6: Mozzicafreddo M, Cuccioloni M, Eleuteri AM, Angeletti M. Rapid reverse phase- HPLC assay of HMG-CoA reductase activity. J Lipid Res. 2010 Aug;51(8):2460-3. doi: 10.1194/jlr.D006155. Epub 2010 Apr 24. PMID: 20418539; PMCID: PMC2903805.



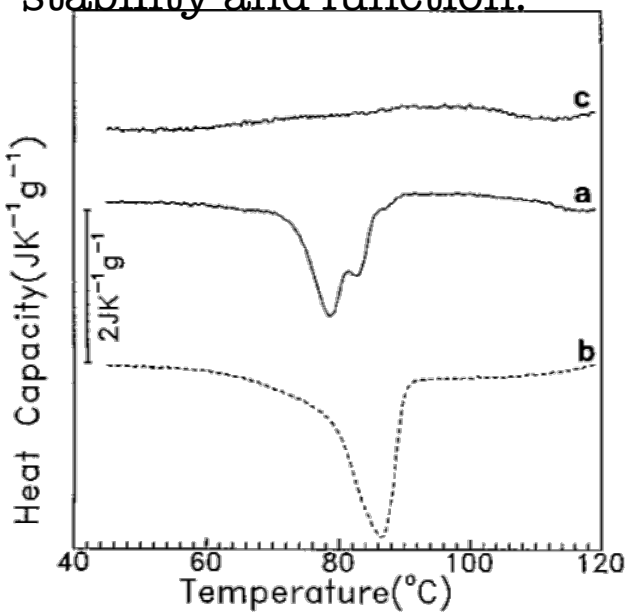
3. Ovalbumin: structure

1. Ovalbumin is the major proteic component of chicken egg white.
2. It is present in several isoforms (PTMs).
3. Under specific pH conditions (pH>8, temperature >50C, slow cooling) can be isomerized to S-Ovalbumin and I-Ovalbumin, having different structure, temperature stability and function.

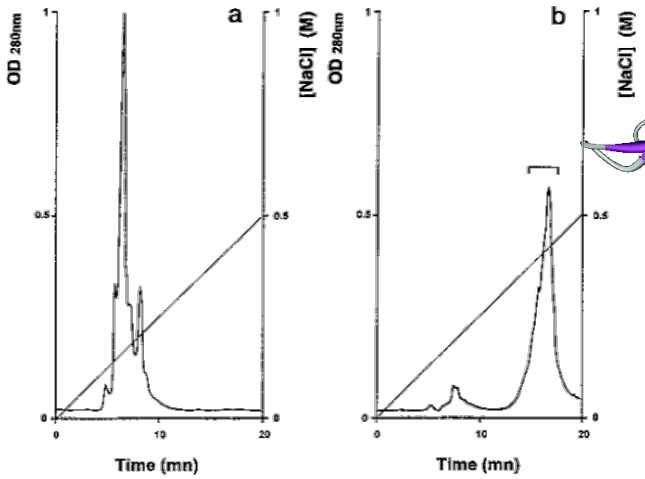
Composition and Physical Properties of Egg White Proteins [16]

	Content % of total protein	Isoelectric point	Molecular weight ($\times 10^4$)
Ovalbumin	54	4.5	4.5
Ovotransferrin	12	6.1	7.6
Ovomucoid	11	4.1	2.8
Ovomucin	3.5	4.5-5.0	-
Ovoglobulin G2	4	5.5	4.9
Ovoglobulin G3	4	5.8	4.9
Lysozyme	3.4	10.7	1.4
Ovomacroglobulin	0.5	4.5	-
Ovoglycoprotein	1	3.9	2.4
Flavoprotein	0.8	4	3.2
Ovoinhibitor	1.5	5.1	4.9
Cystatin (Ficin-papain inhibitor)	0.05	~5.1	1.3
Avidin	0.5	10	6.8

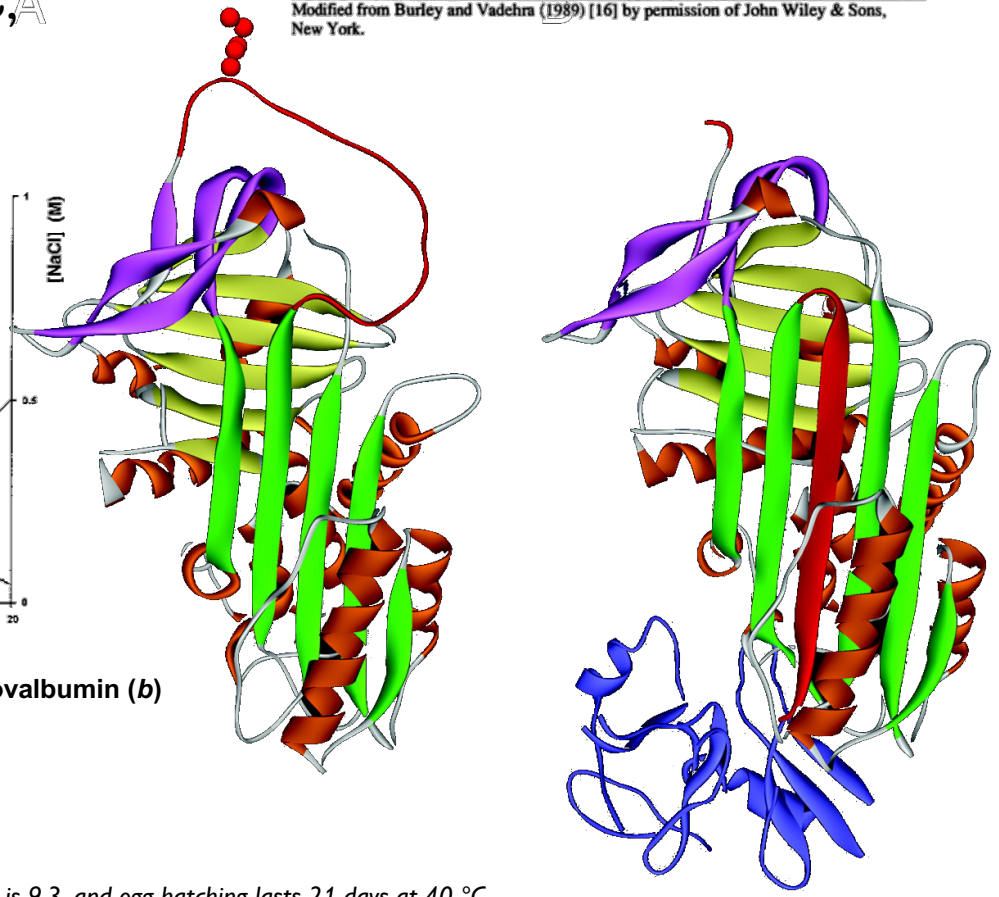
Modified from Burley and Vadehra (1989) [16] by permission of John Wiley & Sons, New York.



Differential scanning microcalorimetry of ovalbumin conformers. The thermograms of native ovalbumin (*a*), S-ovalbumin (*b*), and I-ovalbumin (*c*) have been superimposed.

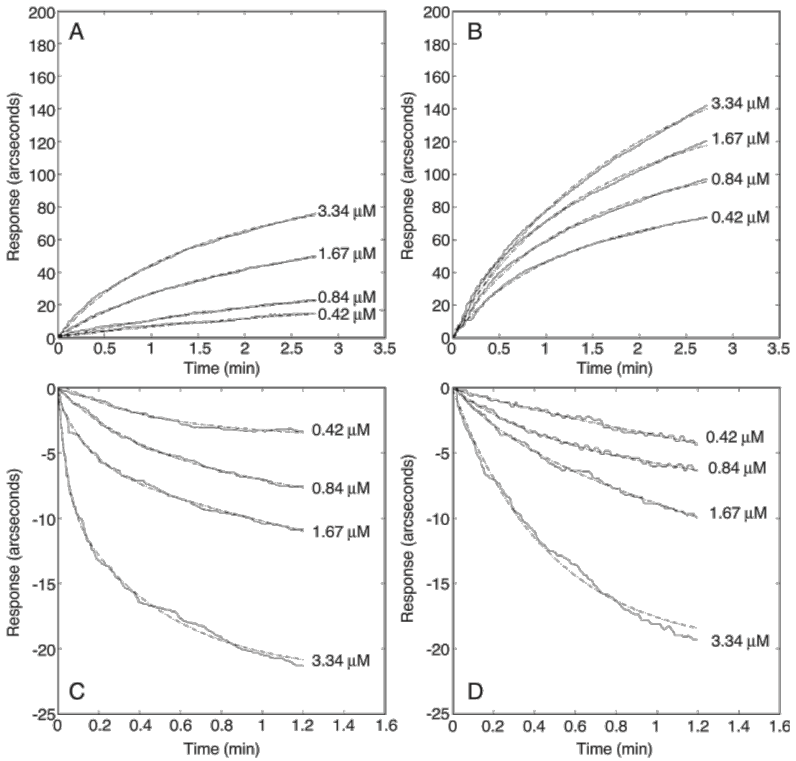


Anion exchange HPLC of native **ovalbumin (*a*), I-ovalbumin (*b*)**



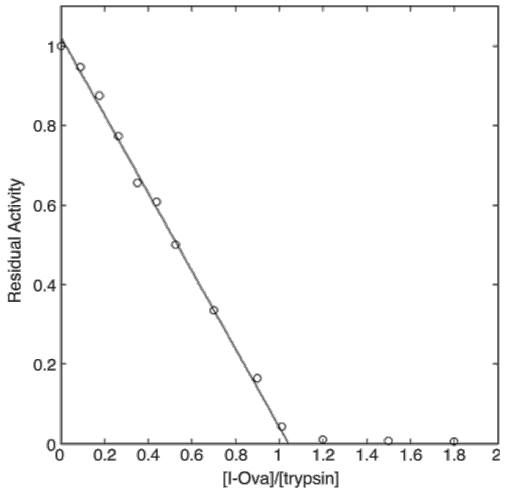
The pH of the egg is 9.3, and egg hatching lasts 21 days at 40 °C

3. I-Ovalbumin: function

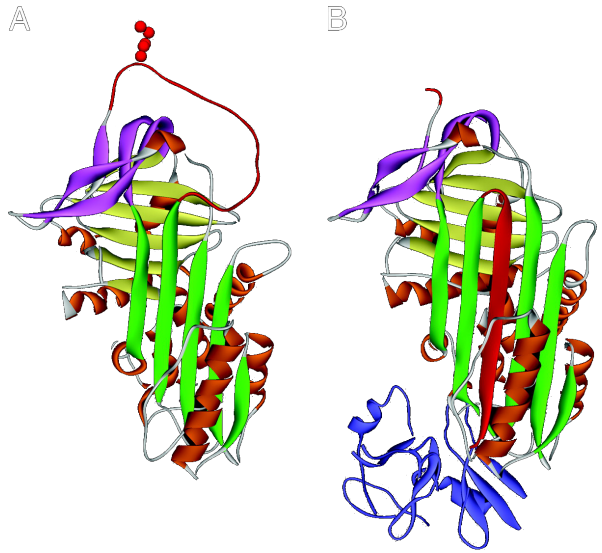


I-ova binding to immobilized trypsin: overlay of association and dissociation phases (solid line) measured at increasing I-ovalbumin concentrations, and the corresponding theoretical curves (dash-dotted line) fitted using Eq. (3) for associations, and Eq. (5) for dissociations (pH=6.2 (A and C), pH=7.4 (B and D)).

Proteinase	K_i
	M
Human neutrophil elastase	$(5 \pm 0.5) \times 10^{-9}$
Bovine pancreatic α-chymotrypsin	$(3 \pm 0.3) \times 10^{-8}$
Human neutrophil cathepsin G	$(6 \pm 2) \times 10^{-8}$
Bovine pancreatic trypsin	$(8 \pm 0.7) \times 10^{-7}$
Porcine pancreatic elastase	$(2 \pm 0.2) \times 10^{-6}$
α-Lytic protease	$(1 \pm 0.2) \times 10^{-6}$
Human thrombin	$\approx 10^{-5}$



Determination of the trypsin–I-ova binding stoichiometry using L- BAPNA and a high concentration of trypsin (8 microM).



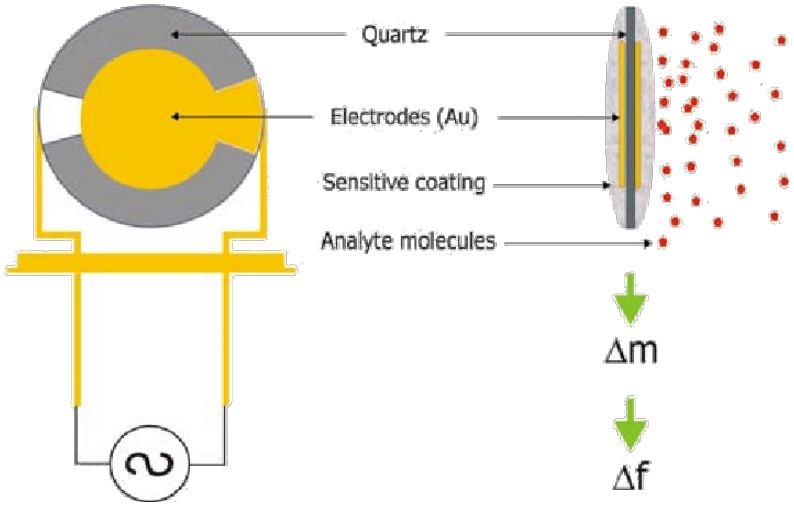
I-Ovalbumin is a potent protease inhibitor, with a 1:1 stoichiometry

I-Ova inhibits HLE with a K_d = 5 nM

Anyway I-Ova binds serine proteases with different affinities (10^{-6}-10^{-9} M range)

Strategies:

- *DSC*
- *Enzymatic assays*
- *kinetics on SPR e QCM (quartz crystal microbalance) biosensors*
- *Docking*



Kinetic and equilibrium characterization of the interaction between bovine trypsin and I-ovalbumin.Cuccioloni M, Sparapani L, Amici M, Lupidi G, Eleuteri AM, Angeletti M.Biochim Biophys Acta. Nov 1;1702(2):199-207.doi:10.1016/j.bbapap.2004.08.019.PMID: 15488772

Peter Privalov, Johns Hopkins University

Research Group

- Laura Bonfili
- Valentina Cecarini
- Massimiliano Cuccioloni
- Anna Maria Eleuteri
- Mauro Angeletti