Ingeniería de Proteínas
o
Optimización de Enzimas

Para que?

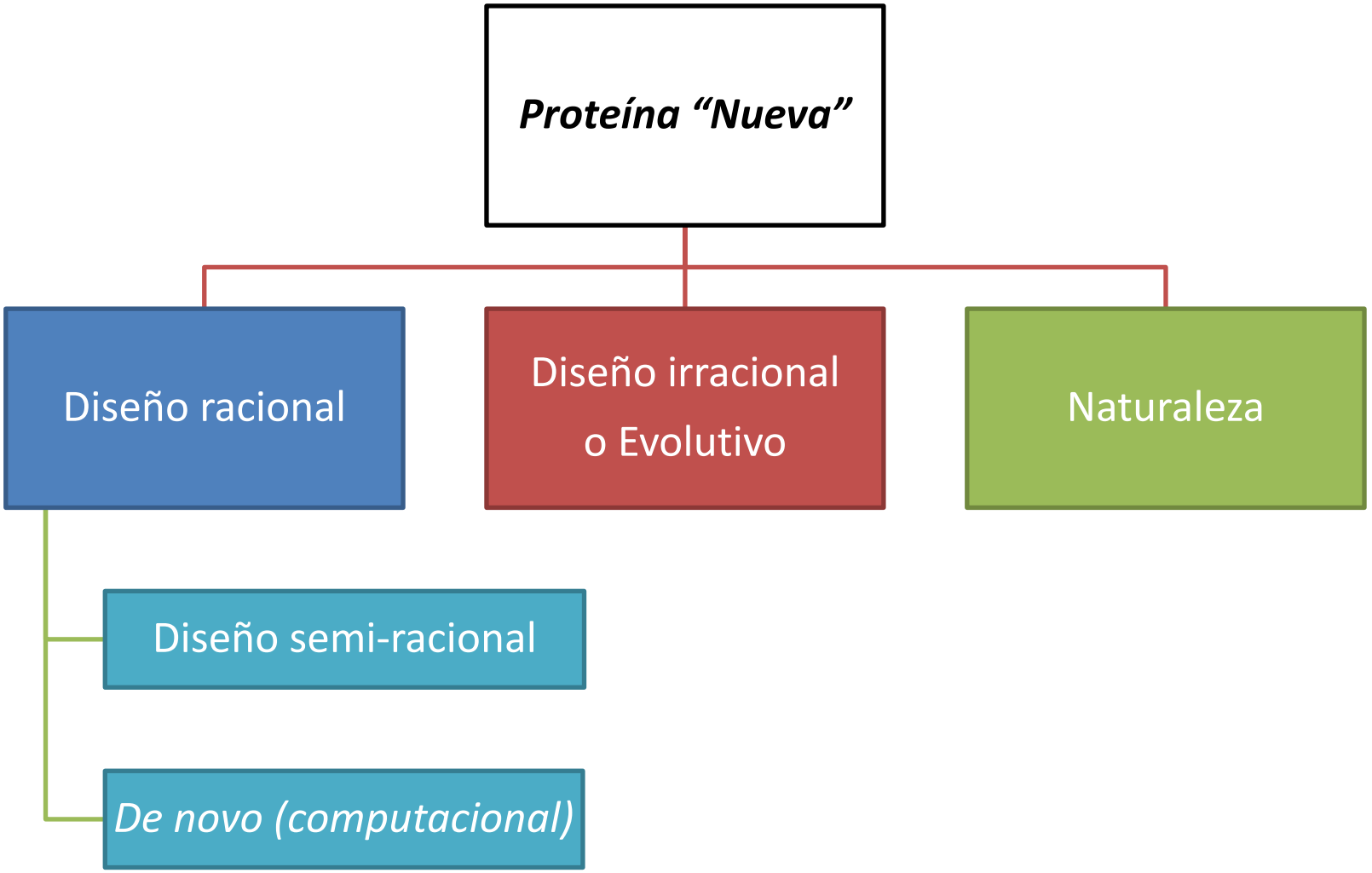
- Mejorar la estabilidad y/o función bajo nuevas condiciones
  - Temperatura, pH, disolvente (orgánico/acuoso), [sal]
- Modificación de la especificidad del sustrato enzimático
- Mejorar la propiedades cineticas
  - Km, Vmax, Allosteric regulation, Cofactor requirements
- Alterar las propiedades de unión

Para que?

Objetivos de la ingeniería de proteínas:

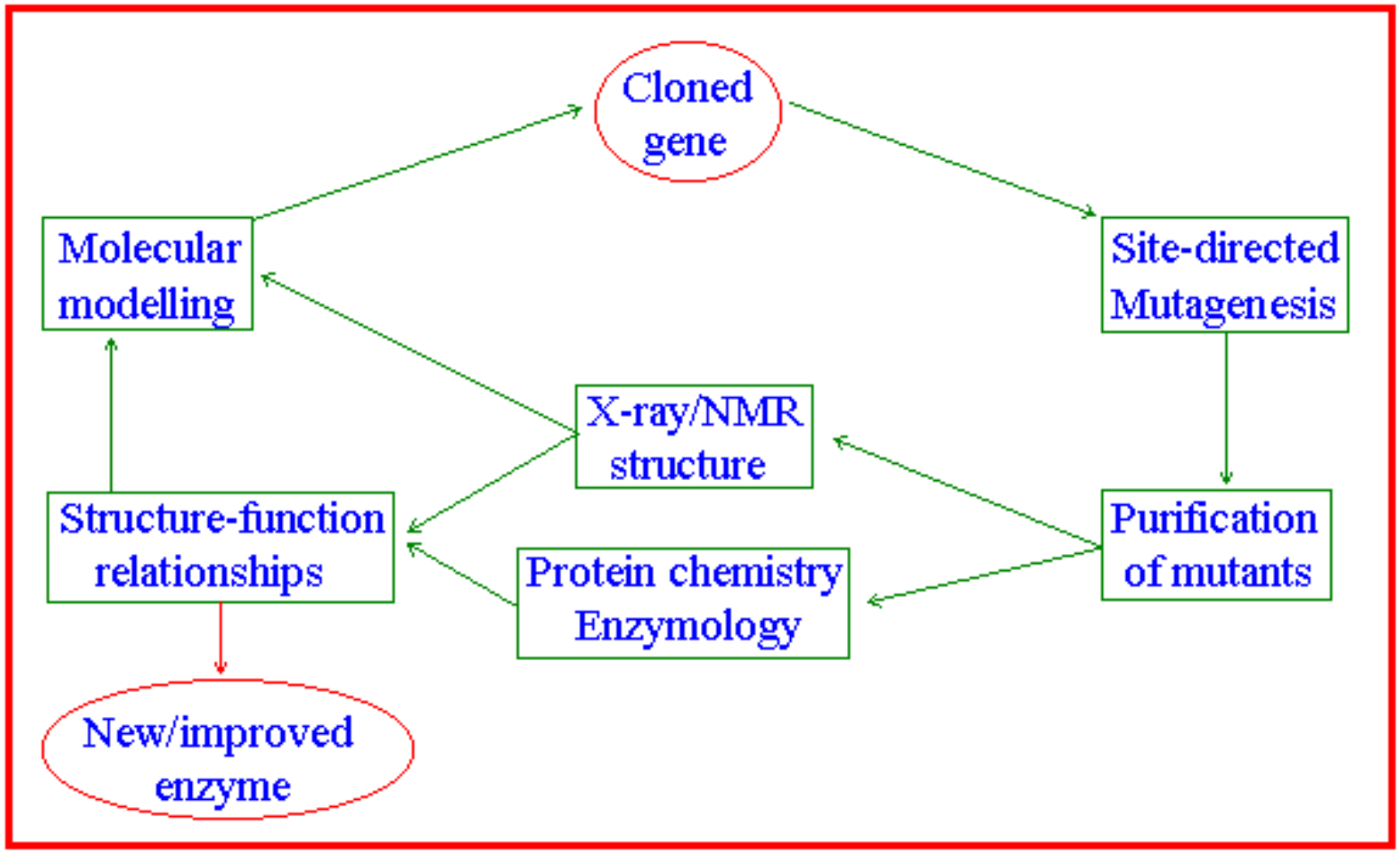
- producir enzima en grandes cantidades
- para crear una enzima superior para catalizar la producción de productos químicos específicos de alto valor
- para producir compuestos biológicos (incluidos péptidos sintéticos, proteínas de almacenamiento y fármacos sintéticos) superiores a los naturales.

Como?


Proteína "Nueva"
Diseño racional
Diseño irracional o Evolutivo
Naturaleza
Diseño semi-racional
De novo (computacional)

Diseño racional

Esquema general



Mutagénesis dirigida

- Mutar AA con posible influencia en propiedad deseada
- Si es posible, mutar AA análogos según otros miembros de la familia
- Es una estrategia de prueba y error...

Diseño racional

- **Mutagénesis dirigida**: métodos

(a) In vitro mutagenesis using synthetic oligonucleotides (*normal lab*)

(b) Synthesis of complete modified gene *de novo* (*companias especializadas*)

Diseño racional

- **Que modificar?**
  1. Agregar cisteinas para inducir puentes disulfuro: puede mejorar estabilidad a expensas de función
  2. Agregar o remover Puentes de Hidrogeno
  3. Remover Asparagina y/o glutamina: evita deaminacion a altas temperatura
  4. Disminuir sulfidrilos libres (por serinas?)
  5. Cambiar requerimiento de cofactores (metales)
  6. Lisina a glutamina: mayor resistencia proteasa

Adding disulfide bonds

- There is generally a direct correlation between the number of disulfide bonds and the thermo-stability of a protein.
- To increase stability of a variety of enzymes disulfide bonds have been added to their structure.
- The problem is whether extra disulfide bonds perturb the normal function.

Native protein

Engineered protein

Adding disulfide bonds

T4 lysozyme

- 6 variants were created using oligonucleotide-directed mutagenesis of amino acid whose R-groups were in close proximity to incorporate more cysteine residues.
- The variants were tested and were generally found to be more thermostable.
- Some variants however lacked activity.
- It is a trial-and-error process.
- However, it is clear that increasing disulfide bonds to enhance protein stability is feasible.

Adding disulfide bonds

T4 lysozyme

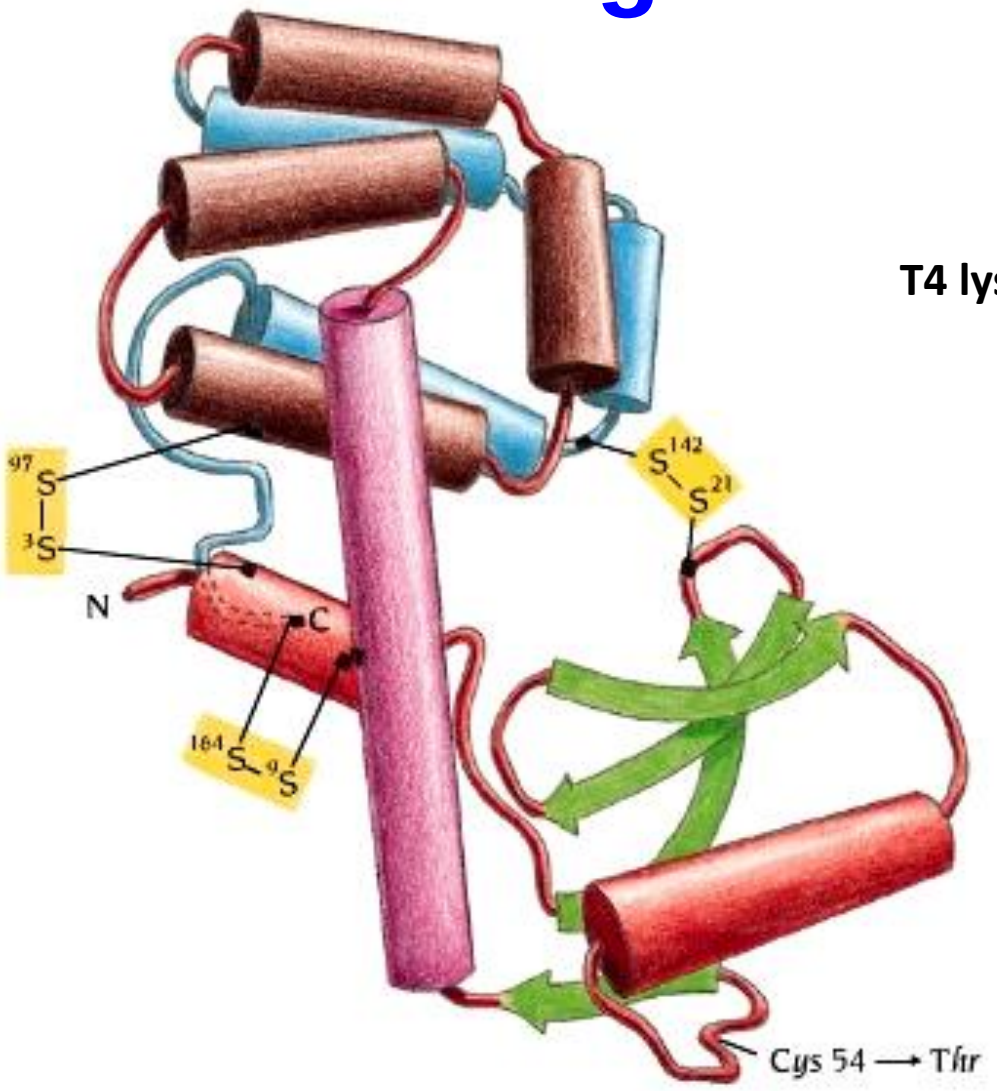
TABLE 8.2 Properties of T4 lysozyme and six engineered variants

Enzyme	Amino acid at position:							No. of -S-S-	% Activity	T_m (°C)
	3	9	21	54	97	142	164			
wt	Ile	Ile	Thr	Cys	Cys	Thr	Leu	0	100	41.9
pwt	Ile	Ile	Thr	Thr	Ala	Thr	Leu	0	100	41.9
A	Cys	Ile	Thr	Thr	Cys	Thr	Leu	1	96	46.7
B	Ile	Cys	Thr	Thr	Ala	Thr	Cys	1	106	48.3
C	Ile	Ile	Cys	Thr	Aka	Cys	Leu	1	0	52.9
D	Cys	Cys	Thr	Thr	Cys	Thr	Cys	2	95	57.6
E	Ile	Cys	Cys	Thr	Ala	Cys	Cys	2	0	58.9
F	Cys	Cys	Cys	Thr	Cys	Cys	Cys	3	0	65.5

Adapted from Matsumura et al., *Nature* **342**:291–293, 1989.

wt, wild-type T4 lysozyme; pwt, pseudo-wild-type enzyme; A through F, six engineered cysteine variants; -S-S-, disulfide bonds; T_m, "melting" temperature (a measure of thermostability).

Adding disulfide bonds



T4 lysozyme: a model for stability studies

Cysteines were added to areas of the protein in close proximity--disulfide bridges could form

Adding disulfide bonds

Xylanase

- more **thermostable**
- An enzyme used in the degradation of hemi-cellulose, the compound generally darkens paper.
- The use of the variant xylanase that function efficiently at high industrial temperature allows the use of less bleach in paper production.
- Less pollution

Adding disulfide bonds

RNase from bull semen has been used as an antitumorigenic agent.

- It is taken up into tumor cells and decreases protein synthesis by degrading rRNA, thereby blocking protein synthesis and causing cell death.
- The antitumor activity is dependent on the dimeric structure.
- Human antibody against the bull semen Rnase are often produced.
- To eliminate this, human pancreatic RNase has been engineered to increase its functional life and activity using disulfide bonds.

Changing Asparagine/Glutamine to Other Amino Acids

- Asparagine and glutamine residues can undergo deamination at high temperature becoming aspartic and glutamic acid, respectively.
- The change could lead to improper folding and loss of enzyme activity.
- A **yeast enzyme** served as the model and showed increased thermostability when Asn was change to threonine and isoleucine.
- Similarly, a modified longer-lasting insulin was produced by changing Asp to Gly (approved for therapeutic use).

Changing Asparagine/Glutamine to Other Amino Acids

TABLE 8.3 Stability at 100°C of the yeast enzyme triosephosphate isomerase and its engineered derivatives

Enzyme	Amino acid at position:		Half-life (min)
	14	78	
Wild type	Asn	Asn	13
Variant A	Asn	Thr	17
Variant B	Asn	Ile	16
Variant C	Thr	Ile	25
Variant D	Asp	Asn	11

Adapted from Ahern et al., *Proc. Natl. Acad. Sci. USA* **84**:657–679, 1987.

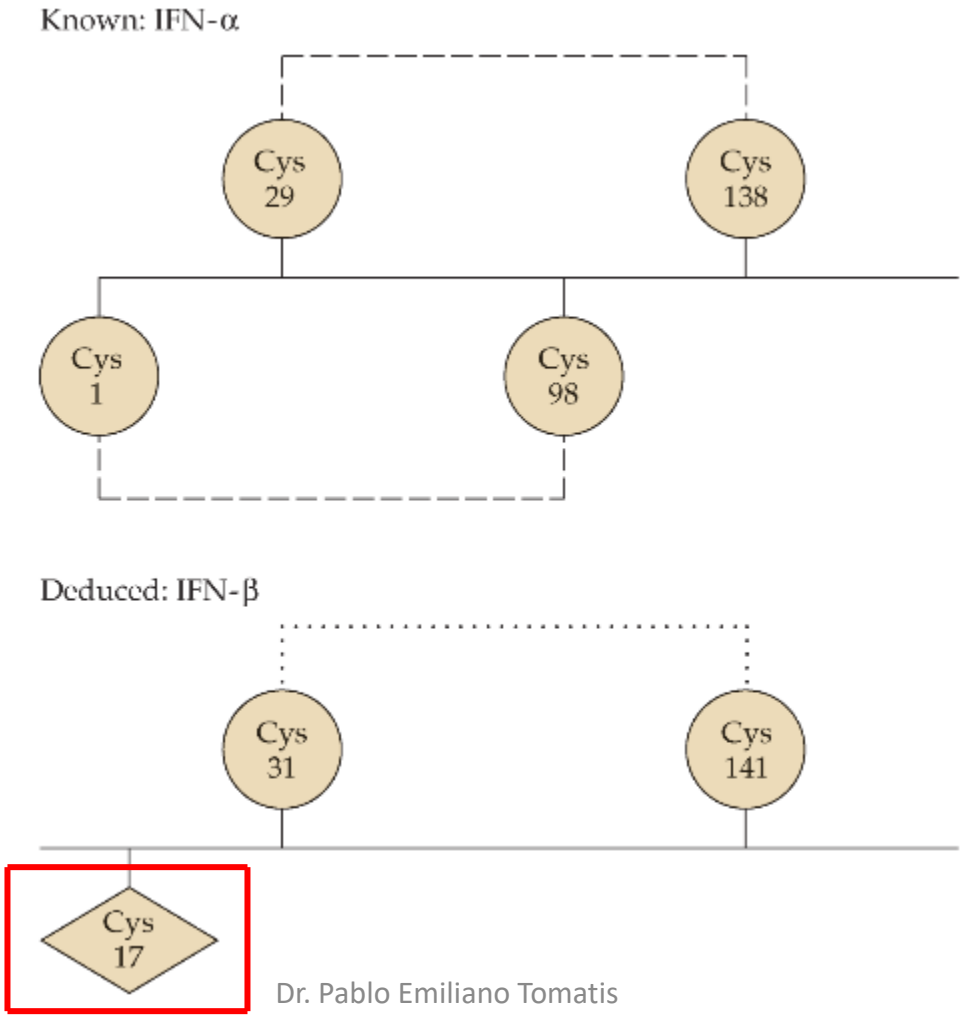
Enzyme stability is expressed as the half-life, or rate of enzyme inactivation, at 100°C. A longer half-life indicates a more stable enzyme.

Reducing the Number of Free Sulfhydryl Residues

Interferon

- In many early expression studies, the expressed protein showed much less activity than would have been predicted.
- Interferon showed only 10% of the antiviral activity of authentic glycosylated form.
- In *E. coli* production, the unglycosylated protein exist as dimers and higher oligomers that were inactive.
- Activity was greatly increased by changing different cysteines to serine.

Reducing the Number of Free Sulfhydryl Residues



Increasing Enzymatic Activity

- Experiments were performed using an **Tyrosyl-tRNA synthetase** for which the active site was well characterized.
- A threonine at position 51 of the protein was targeted for modification.
- If it was replaced with an alanine, the Km of the enzyme increased 2X without altering the rate.
- If it was replaced with proline, the enzyme bound ATP 100X more tightly than normal and increased its efficiency.

Increasing Enzymatic Activity

TABLE 8.4 Aminoacylation activity of native (Thr-51) and modified (Ala-51 and pro-51) tyrosyl-tRNA synthetases

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
Thr-51	4.7	2.5	1,860
Ala-51	4.0	1.2	3,200
Pro-51	1.8	0.019	95,800

Adapted from Wilkenson et al., *Nature* **307**:187–188, 1984.

The units for K_m, the binding constant of the enzyme for ATP, are millimolar units (mM); the units for k_{cat}, the catalytic rate constant, are reciprocal seconds (s^{-1}); and the units for k_{cat}/K_m, the catalytic efficiency, are s^{-1} M^{-1}.

Modifying Metal Cofactor Requirements

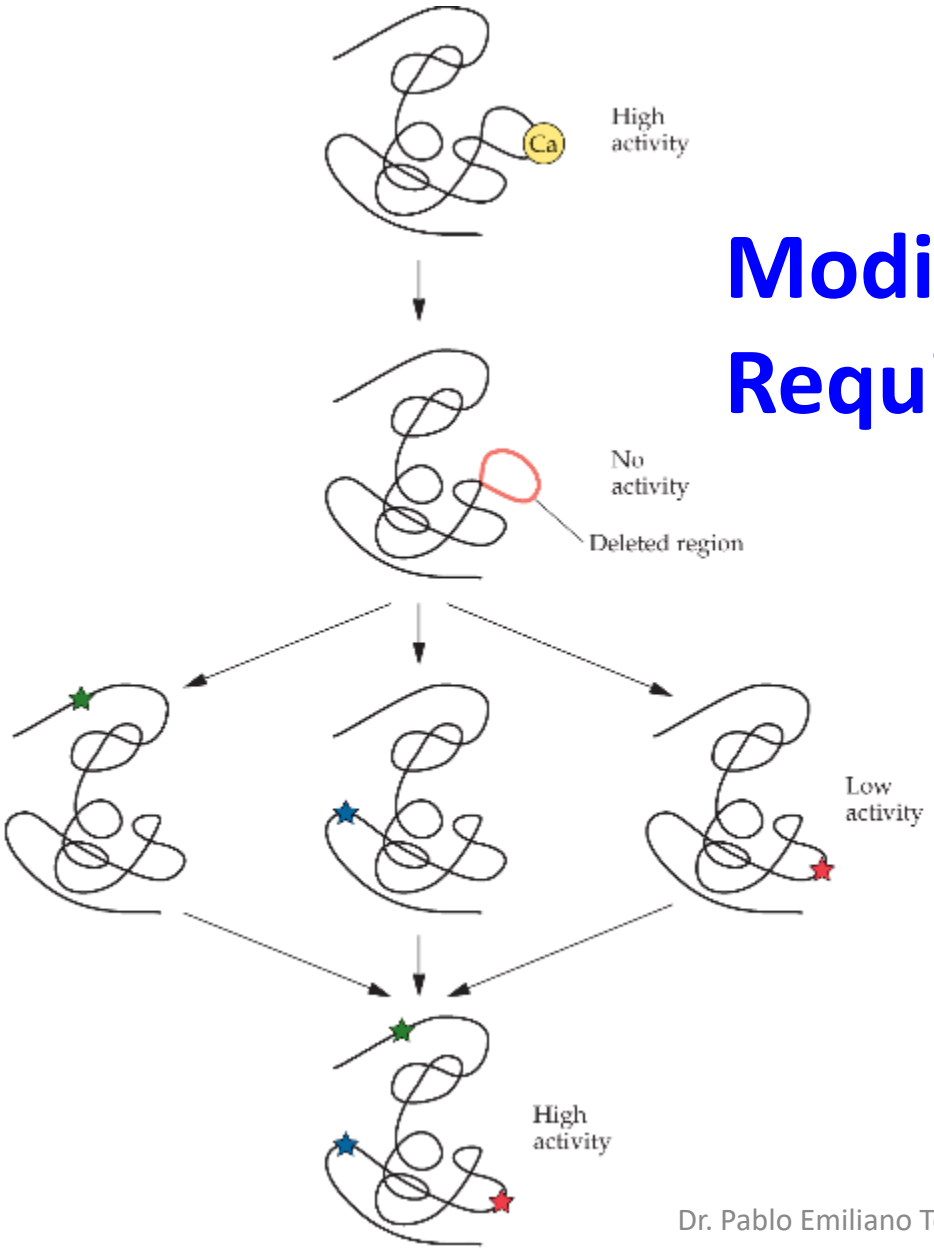
Subtilisin

- This enzyme from gram-positive bacteria and are widely used as biodegradable cleaning agents in laundry detergent.
- It requires **Ca^{+2} as a cofactor** to stabilize the enzyme.
- The enzyme is used in many industrial settings where there are a large number of metal-chelating agents that can bind to and effectively remove calcium.
- These enzymes are rapidly inactivated under these conditions.

Modifying Metal Cofactor Requirements

- To avoid this, the portion of the enzyme required for Ca^{+2} binding was determined and eliminated from the protein (= inactive protein).
- The researchers began changing amino acids to restore enzyme activity.
- The result was a modified subtilisin that retains its activity but no longer requires Ca^{+2} ions for improved industrial use.

Diseño racional



Modifying Metal Cofactor Requirements

Modifying Metal Cofactor Requirements

TABLE 8.5 Effects of random mutations of selected amino acid residues on the stability of subtilisin BPN lacking a calcium-binding domain

Region of protein	Amino acid residue	Stabilizing mutation	Fold increase in half-life
N terminus	2	Gln→Lys	2.0
	3	Ser→Cys	17.0
	4	None found	None
	5	Pro→Ser	1.2
Omega loop	41	Asp→Ala	1.2
	44	Lys→Asn	1.2
α-Helix	73	Ala→Leu	2.6
	74	None found	None
β-Pleat structure	206	Gln→Cys	17.0
	214	None found	None

Adapted from Strausberg et al., *Bio/Technology* **13:** 669–673, 1995.

The mutations at positions 3 and 206 to Cys occur in the same clone and provide such a high level of stability because of the formation of the disulfide bridge between these residues.

Decreasing Protease Sensitivity

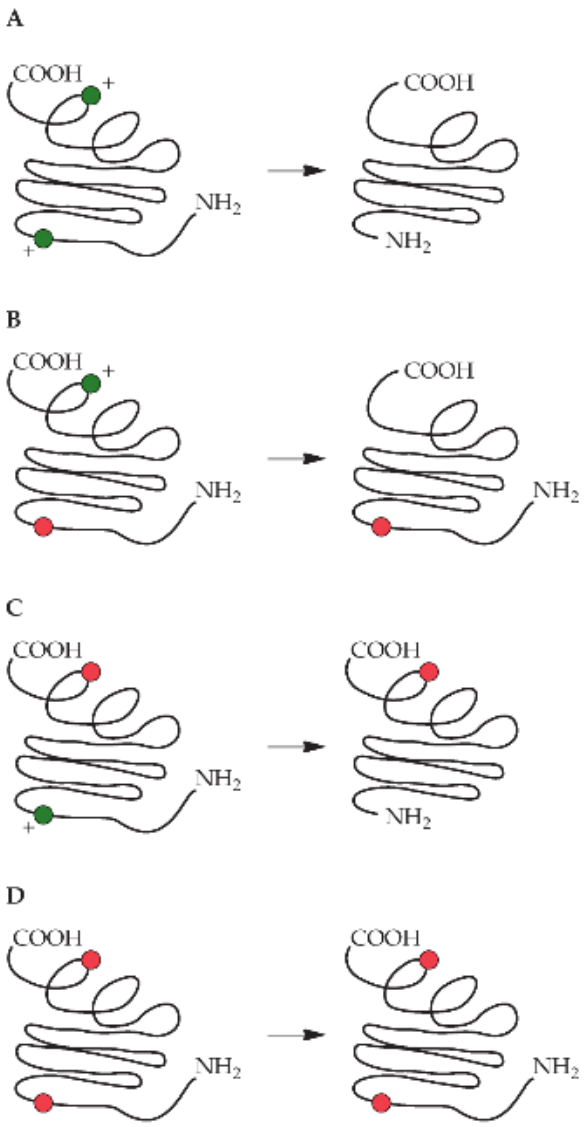
Streptokinase

- a protein produced by pathogenic strain of *Streptococcus* bacteria that is a blood clot dissolving agent.
- The protein complexes with plasminogen converting it to plasmin; the protease that degrades the fibrin in a blood clot.
- Unfortunately, plasmin also degrades streptokinase decrease its useful life in medical treatment.
- A long-lived streptokinase could be administered as a single injection before a person with heart attacked is transported to a hospital.

Decreasing Protease Sensitivity

- Plasmin is a trypsin-like protease cleaving the peptide bond adjacent to a lysine or arginine residue.
- Plasmin cleaves streptokinase twice after 2 separate lysine residues, lysine 59 and lysine 386.
- To make streptokinase less susceptible to plasmin, the two lysine residues were changed to glutamine by oligonucleotide-directed mutagenesis.
- Glutamine does not have positive charge and the length of its side chain is similar to lysine thus not changing the three dimensional structure.
- The double mutant showed **21X more protease resistance.**

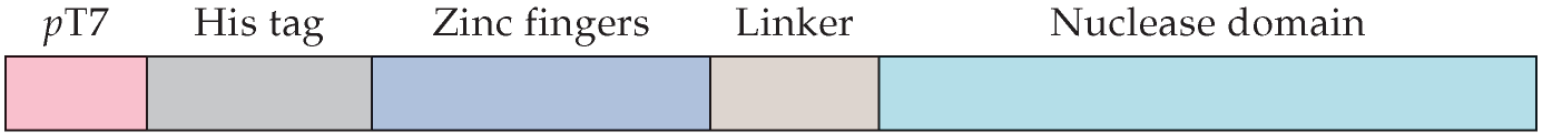
Diseño racional



Processed size (kDa)	Activity
37	Low
42	Low
40	Low
47	High

Modifying Protein Specificity

- *Fok*I restriction enzyme from *Flavobacterium okeanokoites* was used as a model. It is a relatively non-specific nuclease.
- To add specificity to the enzyme, the portion of the gene encoding the catalytic domain was fused to another segment of DNA encoding histidine, three zinc finger domains, a linker peptide to confer flexibility.
- The modified enzyme was shown to recognize the expected site and to a lesser degree another two sites.



Diseño racional

- modify protein function based on understanding consequences of certain changes
- We are still relatively ignorant as to how a protein´s gene sequence encodes functionality

Directed evolution avoids this problem by creating libraries of variants possessing desired properties