Enzyme and Fermentation Technology:

"Part 2: Enzyme Engineering"

Associate Professor:

Dr. I-Son Grace Ng

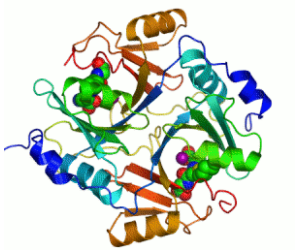
吳意珣 博士

化學工程學系
國立成功大學

What's an enzyme?

- ◆Classification
- ◆Characteristic
- ◆ Enzymatic Unit
- ◆Parameters involved in activity
- ◆Commercial enzymes

What's an enzyme?



- A functional protein called as an enzyme.
- They are responsible for thousands of metabolic processes that sustain life.

1 酵素的命名

Trypsin Pepsin Renin Lysozyme

胰蛋白酶 胃蛋白酶 腎蛋白酶 溶菌酶

催化反應 -ase

EC 4.1.1.22 →

Enzyme Commission

Histidine 基質
carboxylase 反應

4 Main Class	Lyase
1 Subclass	C-C lyase
1 Sub-subclass	carboxylase
22 Series number	第 22 個

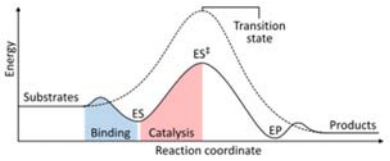
Juang RH (2007) BCha

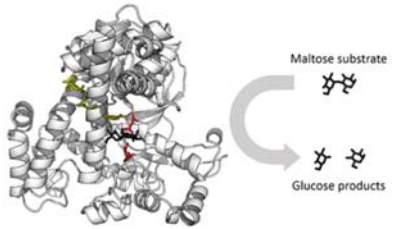
Enzyme classification

Group	Reaction catalyzed	Typical reaction	Enzyme example(s) with trivial name
EC 1 *Oxidoreductases*	To catalyze oxidation/reduction reactions; transfer of H and O atoms or electrons from one substance to another	AH + B → A + BH (**reduced**) A + O → AO (**oxidized**)	Dehydrogenase, oxidase
EC 2 *Transferases*	Transfer of a functional group from one substance to another. The group may be methyl-, acyl-, amino- or phosphate group	AB + C → A + BC	Transaminase, kinase
EC 3 *Hydrolases*	Formation of two products from a substrate by hydrolysis	AB + H_2O → AOH + BH	Lipase, amylase, peptidase
EC 4 *Lyases*	Non-hydrolytic addition or removal of groups from substrates. C-C, C-N, C-O or C-S bonds may be cleaved	RCOCOOH → RCOH + CO_2 or [X-A-B-Y] → [A=B + X-Y]	Decarboxylase
EC 5 *Isomerases*	Intramolecule rearrangement, i.e. isomerization changes within a single molecule	ABC → BCA	Isomerase, mutase
EC 6 *Ligases*	Join together two molecules by synthesis of new C-O, C-S, C-N or C-C bonds with simultaneous breakdown of ATP	X + Y+ ATP → XY + ADP + Pi	Synthetase

Enzymes characteristics

- Enzyme, as a protein, is similar to catalyst and so called "biocatalyst".
- Enzyme accelerates the reaction via decreased the activation energy.
- The reaction is under mild condition but concerns optimal pH and temperature.





Glucosidase converts sugar maltose to two glucose.

Enzymes characteristics

- Catalytic power
- High specificity
- Co-enzymes or co-factors

Table 1.1 Advantages and Drawbacks of Enzymes as Catalysts

Advantages	Drawbacks
High specificity	High molecular complexity
High activity under moderate conditions	High production costs
High turnover number	Intrinsic fragility
Highly biodegradable	
Generally considered as natural products	

Enzyme image (印象酵素)

- **Definition:**

  1 Unit of enzymatic activity was defined as the amount of the enzyme that catalyzed the reactant of 1 μmole per minute at optimal temperature, pH with defined volume or enzyme amount.

- 酶活定義:

  單位時間、單位體積在最佳溫度、最佳pH條件下，反應消耗或生成1 μmole的反應物或產物，定義為1單位 (1 unit)

- 比酶活: 每 mg酵素的反應速度定義為 U/mg

Calculation of enzymatic activity: CMCase

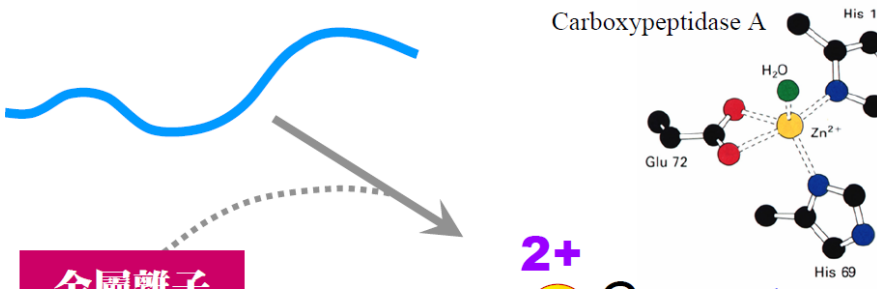
CMCase is also a kind of endo-glucanase or say “cellulase”:

1. Enzyme activity toward CMC was measured according to dinitrosalicylic acid (DNS) method (Miller 1959).
2. The 200 µl reaction mixture in 0.5 ml tube contained 50 mM phosphate buffer (pH 7.0), 0.5% CMC and 50 µl enzyme solution. After incubation at 55°C in a thermal cycle machine for 10 min.
3. The concentration of reducing sugars was determined by measuring OD_{540} using the DNS method. One unit of enzyme activity corresponds to 1 µmol glucose per minute in the reaction.
4. FPase assays were carried out with 0.5% Filter paper in place of CMC as substrate. The time of reaction was 30 min.

Other parameters involved in activity

- Metal ion effect
- Co-enzyme and co-factor
- Enzyme Coupling and regeneration
- Key and lock: antigen and antibody

金屬離子可維持蛋白質分子構形



那些胺基酸可與金屬離子結合？

部份蛋白質結構要再加上某些金屬離子

輔酶 NADH 的吸光特性

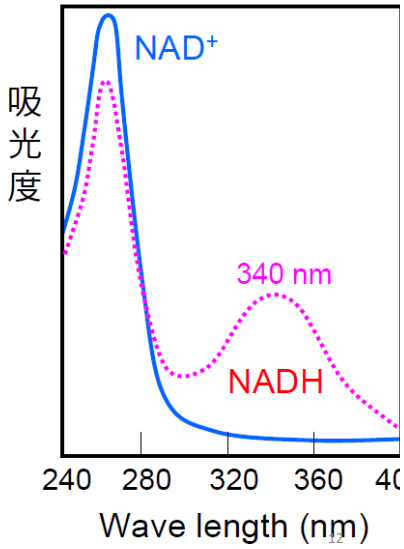
NAD^+/NADH 的轉換可以耦合 340 nm 吸光度變化：

$NAD^+ \rightleftharpoons NADH$ 340 nm

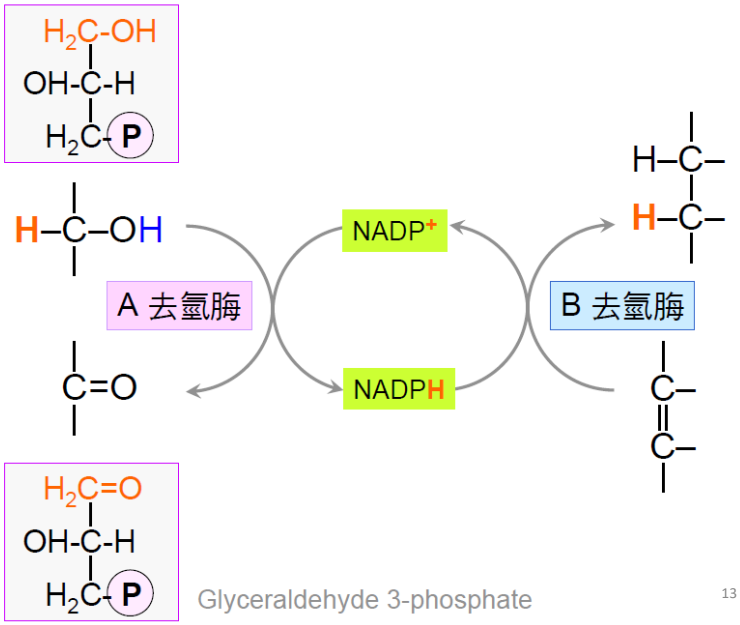
Dehydrogenase (去氫酶)

Glyceraldehyde-3-P deHase

- 所有的 去氫酶 都以 NADH 或 NADPH 為 輔酶
- 都有相似的 NAD^+ 結合 domain (同源演化)

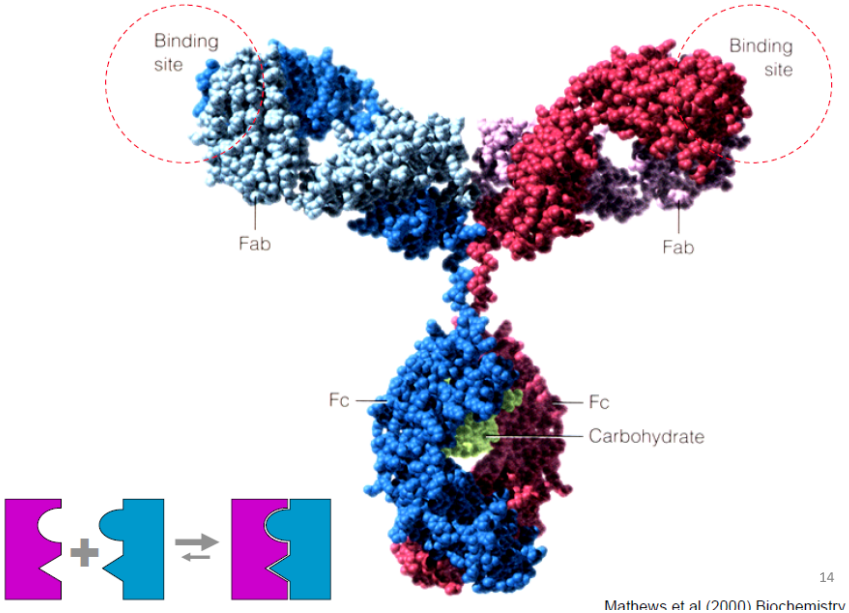


NADH 可耦合氧化及還原反應



Glyceraldehyde 3-phosphate

抗體可專一性地結合抗原

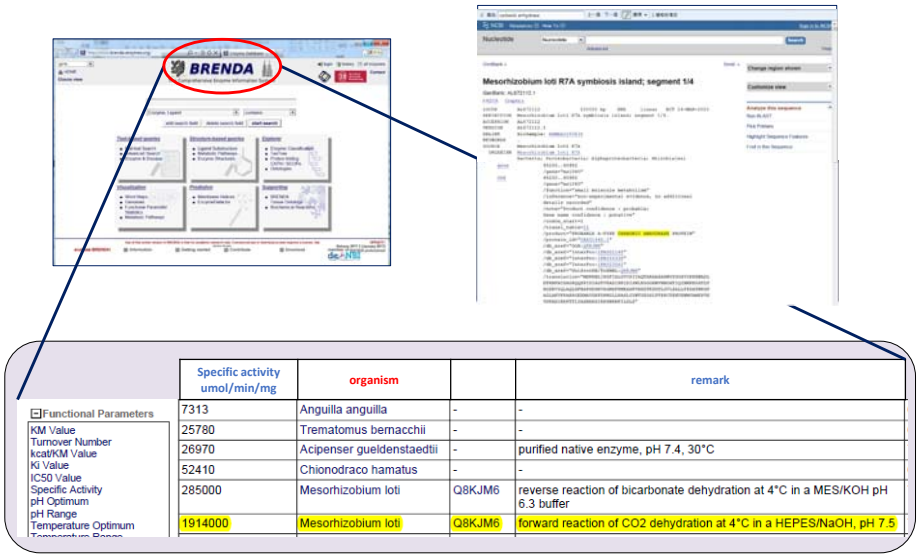


Mathews et al (2000) Biochemistry (3e) p

BRENDA

- The largest bank for enzyme's information.
- http://www.brenda-enzymes.org/index.php
- Find out an enzyme's properties you have interested via BRENDA!

Find out the suitable enzyme candidate from BRENDA



Application

- 酵素市場主要主要範圍為：

1. 工業用酵素：清潔用酵素、紡織及皮革製造用酵素、紙漿加工用酵素。

2. 食品用酵素：澱粉加工製糖用酵素、烘焙釀造、乳製品、果汁及酒類製造純化用。

3. 飼料用酵素：提高組成份的可消化性、增加營養價值及動物產品產量。

Major enzymes in food market

- 主要的食品用酵素有澱粉酵素（amylases）、glucose oxidase（葡萄糖氧化酵素）、chymosin（凝乳酵素）、phytase（植酸酵素）及glucose-isomerases.
- Glucose-isomerases是使用在澱粉液化（liquefaction）、糖化（saccharification）及異構反應（isomerization reaction）。
- 澱粉加工用酵素需克服的包括在加工過程的低催化活性、耐熱性低及高製造成本。

Novozymes ®

ENZYME CLASS	INDUSTRIAL ENZYMES
EC 1: Oxidoreductases	catalase
	glucose oxidase
	laccase
EC 2: Transferases	glucosyltransferase
EC 3: Hydrolases	amylase
	cellulase
	lipase
	mannanase
	pectinase
	phytase
	protease
	pullulanase
	xylanase
EC 4: Lyases	pectate lyase
	alpha-acetolactate decarboxylase
EC 5: Isomerases	glucose isomerase
EC 6: Ligases	not used at present

Table 1. A selection of enzyme types used in industrial processes. (The classes are defined in Table 2.)

novozymes
Rethink Tomorrow

Enzymes for detergency

1. A better cleaning performance in general
2. Rejuvenation of cotton fabric through the action of cellulases on fibers
3. Reduced energy consumption by enabling lower washing temperatures
4. Reduced water consumption through more effective soil release
5. Minimal environmental impact since they are readily biodegradable
6. Environmentally friendlier wash water effluents
7. (in particular, phosphate-free and less alkaline)

4. Enzymes for detergency and personal care

Enzymes application in non-food industry

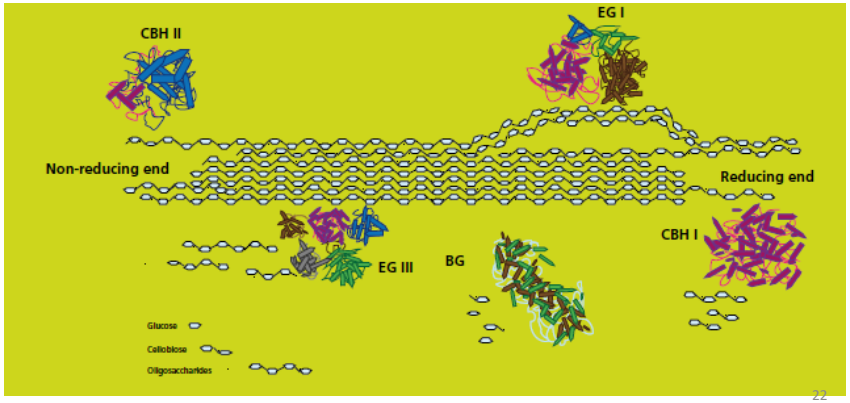
1. Laccase
2. Cellulase
3. Leather-making
4. .....

Amylases	Starch modification Deinking Drainage improvement Cleaning
Xylanases	Bleach boosting Energy saving
Cellulases	Deinking Drainage improvement Energy saving Tissue and fiber modification
Lipases and esterases	Pitch control Stickies control Deinking Cleaning

Table 3. Examples of enzyme applications in the pulp and paper industry.

Enzymes application in Energy

1. Endoglucanase (EG)
2. Exoglucanase or cellobiohydrolase (CBH)
3. Beta-glucosidae (BG)



Enzymes application in food industry

ENZYME	EFFECT
Amylase	Maximizes the fermentation process to obtain an even crumb structure and a high loaf volume
Maltogenic alpha-amylase	Improves shelf life of bread and cakes through antistaling
Glucose oxidase	Cross-links gluten to make weak doughs stronger, drier and more elastic
Lipase	Modifies the natural lipids in flour to strengthen the dough
Lipoxygenase	Bleaches and strengthens dough
Asparaginase	Reduces the amount of acrylamide formed during baking

1. Sugar Processing
2. Saccharification
3. Baking (yeast)

Major enzymes provided by Novozymes®

ENZYME	SUBSTRATE	PRODUCT	APPLICATION
nitrile hydratase	pyridine-3-carbonitrile	nicotinamide	Pharmaceutical intermediate
nitrile hydratase	acrylonitrile	acrylamide	Intermediate for water-soluble polymers
D-amino acid oxidase & glutaryl acylase	cephalosporin C	7-aminocephalosporanic acid	Intermediate for semisynthetic antibiotics
penicillin acylase	7-aminodeacetoxy-cephalosporanic acid	cephalexin	Antibiotics
penicillin G acylase	penicillin G	6-aminopenicillanic acid	Intermediate for semisynthetic antibiotics
ammonia-lyase	fumaric acid + ammonia	L-aspartic acid	Intermediate for aspartame
thermolysin	L-aspartic acid + D,L-phenylalanine	aspartame (after methylation)	Artificial sweetener
dehalogenase	(*R*,*S*)-2-chloropropionic acid	(*S*)-2-chloropropionic acid	Intermediate for herbicides
lipase	(*R*,*S*)-glycidyl butyrate	(*S*)-glycidyl butyrate	Chemical intermediate
lipase	isosorbide diacetate	isosorbide 2-acetate	Pharmaceutical intermediate
lipase	(*R*,*S*)-naproxen ethyl ester	(*S*)-naproxen	Drug
lipase	racemic 2,3-epoxy-3-(4-methoxyphenyl)propionic acid methyl ester	(2*R*,3*S*)-2,3-epoxy-3-(4-methoxyphenyl)propionic acid methyl ester	Pharmaceutical intermediate
acylase	D,L-valine + acetic acid	L-valine	Pharmaceutical intermediate
acylase	acetyl-D,L-methionine	L-methionine	Pharmaceutical intermediate

Catalysis and Biocatalysis

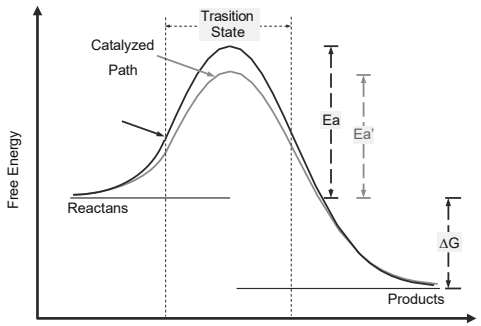


Fig. 1.1 Mechanism of catalysis. Ea and Eat are the energies of activation of the uncatalyzed and catalyzed reaction. DG is the free energy change of the reaction

Table 1.1 Advantages and Drawbacks of Enzymes as Catalysts

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Enzymes as Catalysts. Structure–Functionality Relationships

- Every protein is conditioned by its amino acid sequence, called *primary structure*, which is genetically determined by the deoxyribonucleotide sequence in the structural gene that codes for it.
- Synthesized polypeptide chain is transformed into a three-dimensional structure, called *native structure*, which is the one endowed with biological functionality.
- Secondary three-dimensional structure is the result of interactions of amino acid residues proximate in the primary structure, mainly by hydrogen bonding of the amide groups; for the case of globular proteins, like enzymes, these interactions dictate a predominantly ribbon-like coiled configuration termed α-*helix*.
- Tertiary three-dimensional structure is the result of interactions of amino acid residues located apart in the primary structure that produce a compact and twisted configuration in which the surface is rich in polar amino acid residues, while the inner part is abundant in hydrophobic amino acid residues.

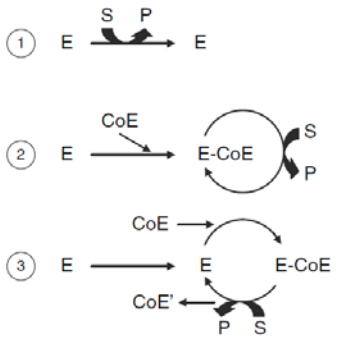
Main types of interactions responsible for the three-dimensional structure

- Hydrogen bonds, resulting from the interaction of a proton linked to an electronegative atom with another electronegative atom. A hydrogen bond has ap- proximately one-tenth of the energy stored in a covalent bond. It is the main determinant of the helical secondary structure of globular proteins and it plays a significant role in tertiary structure as well.
- Apolar interactions, as a result of the mutual repulsion of the hydrophobic amino acid residues by a polar solvent, like water. It is a rather weak interaction that does not represent a proper chemical bond (approximation between atoms exceed the van der Waals radius); however, its contribution to the stabilization of the three- dimensional structure of a protein is quite significant.

- Disulphide bridges, produced by oxidation of cysteine residues. They are especially relevant in the stabilization of the three-dimensional structure of low molecular weight extracellular proteins.
- Ionic bonds between charged amino acid residues. They contribute to the stabilization of the three-dimensional structure of a protein, although to a lesser extent, because the ionic strength of the surrounding medium is usually high so that interaction is produced preferentially between amino acid residues and ions in the medium.
- Other weak type interactions, like van der Waals forces, whose contribution to three-dimensional structure is not considered significant.

Coenzyme mechanism

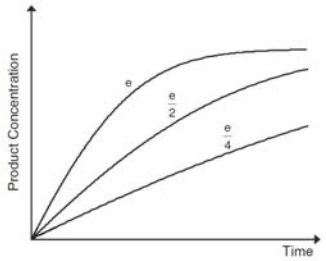
Fig. 1.3 Enzymes according to their cofactor or coenzyme requirements. 1: no requirement; 2: cofactor requiring; 3: coenzyme requiring



Concept and Determination of Enzyme Activity

$$S \xrightarrow{E} P$$

$$v = -\frac{ds}{dt} = \frac{dp}{dt}$$



course of an enzyme catalyzed reaction: product concentration versus time of reac- it enzyme concentrations (e)

- An example of a coupled system considering coenzyme determination is the assay for lactase (β-galactosidase; EC 3.2.1.23). The enzyme catalyzes the hydrolysis of lactose according to:
- Lactose + H2O → Glucose + Galactose
- Glucose produced can be coupled to a classical enzymatic glucose kit, that is: hexoquinase (Hx) plus glucose 6 phosphate dehydrogenase (G6PD), in which:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hx}} \text{Glucose 6Pi} + \text{ADP}$$

$$\text{Glucose 6Pi} + \text{NADP}^{+} \xrightarrow{\text{G6PD}} \text{6PiGluconate} + \text{NADPH}$$

Concept and Determination of Enzyme Activity

- It is based on the absorption of light of a certain wavelength as described by the Beer–Lambert law:

$$A_\lambda = \varepsilon \cdot l \cdot c$$

$$A_\lambda = \log \frac{I}{I_0}$$

For instance, the reduced coenzyme NADH (or NADPH) has a strong peak of absorbance at 340 nm while the absorbance of the oxidized coenzyme NAD+ (or NADP+) is negligible at that wavelength; therefore, the activity of any enzyme producing or consuming NADH (or NADPH) can be determined by measuring the increase or decline of absorbance at 340 nm in a spectrophotometer.

For instance, the activity of glutamate decarboxylase (EC 4.1.1.15), that catalyzes the decarboxylation of glutamic acid to γ-aminobutyric acid and CO2, has been assayed in a differential respirometer by measuring the increase in pressure caused by the formation of gaseous CO2

HPLC applied in enzyme assay

- If both substrate and product absorb significantly at a certain wavelength, coupling the detector to an appropriate high performance liquid chromatography (HPLC) column can solve this interference by separating those peaks by differential retardation of the analytes in the column.
- HPLC systems are increasingly common in research laboratories, so this is a very convenient and flexible way for assaying enzyme activities

Enzyme Unit Definition

• Enzyme activity is expressed in units of activity. The Enzyme Commission of the International Union of Biochemistry recommends to express it in international units (IU), defining 1 **IU as the amount of an enzyme that catalyzes the transformation of 1 µmol of substrate per minute under standard conditions of temperature, optimal pH, and optimal substrate concentration** (International Union of Biochemistry).

- Later on, in 1972, the Commission on Biochemical Nomenclature recommended that, in order to adhere to SI units, reaction rates should be expressed in moles per second and the ***katal* was proposed as the new unit of enzyme activity, defining it as the catalytic activity that will raise the rate of reaction by 1 mol/second** in a specified assay system (Anonymous 1979).

Enzyme Production

Enzyme Sources

Production of Enzymes

Native Production

Recombinant Production

Enzyme Recovery

Enzyme Purification

Enzyme Formulation

Introduction of different kinds enzymes

- Nowadays, enzymes from plants and animals, mostly proteases, are still in the market and some of them are of commercial relevance.
- Catalase (EC 1.11.1.6) from liver (Yildiz et al. 2004), lipase (EC 3.1.1.3), chymotrypsin (EC 3.4.21.1), and trypsin (EC 3.4.21.4) from pancreas (Underkofler et al. 1958) and rennin from calf abomasus are the most relevant animal enzymes, widely used in the food and leather industries (Oberg et al. 1992; Kosikowski and Mistry 1997).
- Animal derived enzymes represent about 10% of the total enzyme market.

Simple Fermentation Process

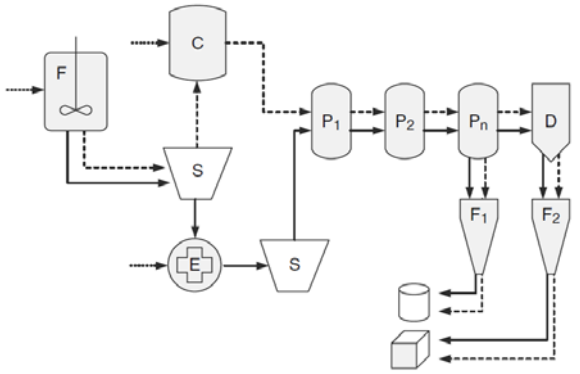
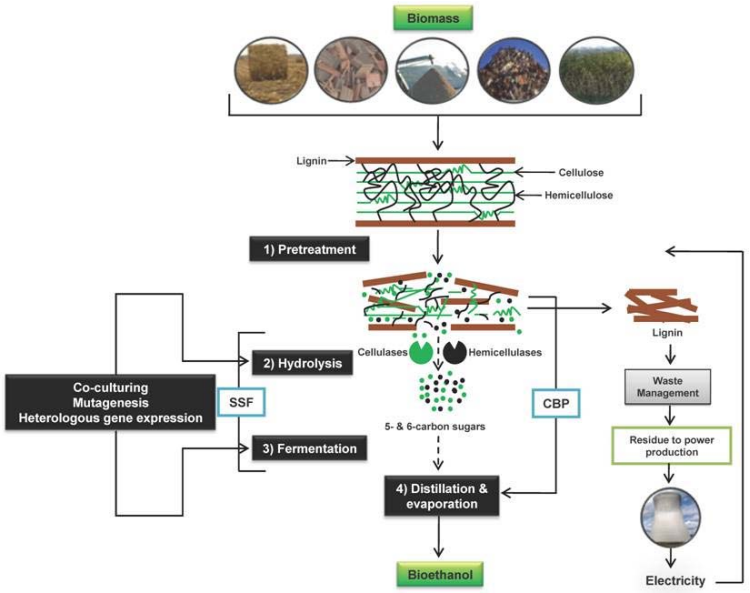
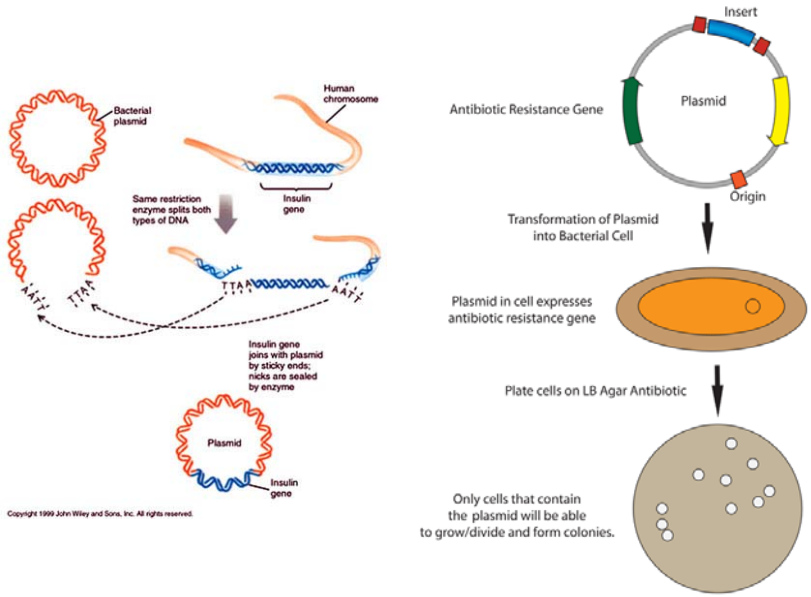


Fig. 2.1 Scheme for the production of enzymes. F: fermentation; S: solid–liquid separation; E: cell extraction; C: concentration; P_i: operations of purification; D: drying; F_i: formulation; - - - ▶: extracellular enzyme; ——▶: intracellular enzyme; cell tissue or fluid - - - ▶

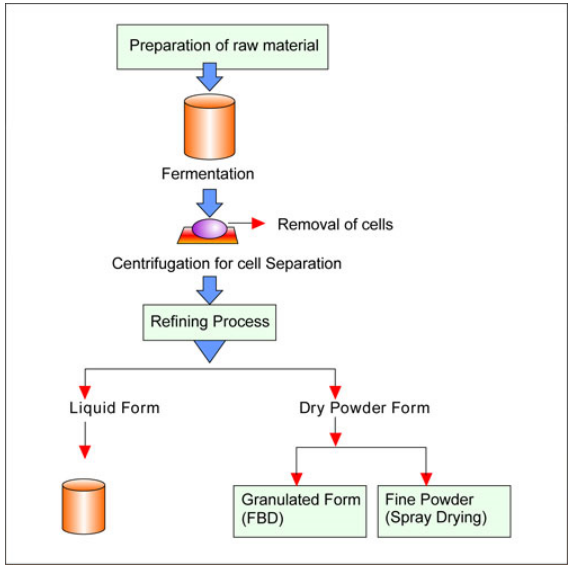
Native enzymes production:
Lignocellulosic enzymes production from waste treatment



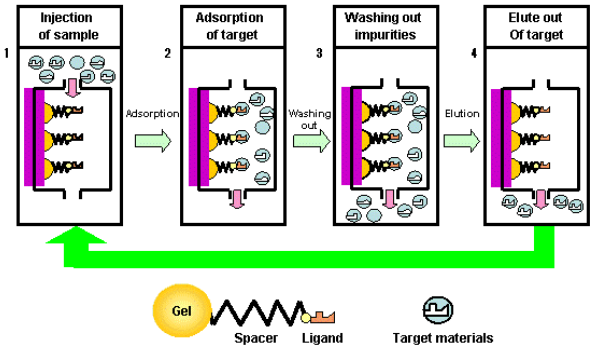
Recombinant Production



Enzyme Recovery



Enzyme Purification



Consideration:
Capacity, cost, purity

Enzyme Formulation

Remove the contamination
Lyophilization
Storage
Liquid or solid form

Green Fluorescent Protein (GFP as a reporter and its application

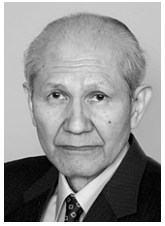
BACKGROUND

- During the 20th century the foundations of biochemistry were laid and used to explore the basal principles of the anabolic and catabolic pathways inside living cells.
- The 20th century also witnessed a revolution in our understanding of enzyme function and through crystallography and nuclear magnetic resonance (NMR), of protein structures revealed at atomic resolution.
- During the second half of this century, classical genetics and nucleic-acid chemistry merged into modern genomics based on whole-genome sequencing of an ever increasing number of organisms.

BACKGROUND

- Neither the biochemical nor the genetics revolution provided the experimental tools that would allow for quantitative and experimentally well-defined monitoring.
- At the beginning of the 21st century, we are witnessing the rapid development of such tools based on the green fluorescent protein (GFP) from the jellyfish *Aequorea vicotria*.

2008 Nobel Prize in Chemistry



Osamu Shimomura
Born: 27 August 1928, Kyoto, Japan
Affiliation at the time of the award: Marine Biological Laboratory (MBL), Woods Hole, MA, USA, Boston University Medical School, Massachusetts, MA, USA
Prize motivation: "for the **discovery of the green fluorescent protein, GFP from Aequorea victoria**"

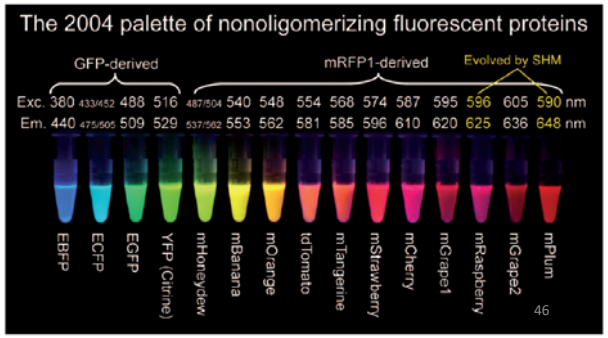
Martin Chalfie
Born: 15 January 1947, Chicago, IL, USA
Affiliation at the time of the award: Columbia University, New York, NY, USA

Prize motivation: "for the **development of the green fluorescent protein, GFP in ELISA technique**"

Roger Y. Tsien
Born: 1 February 1952, New York, NY, USA
Died: 24 August 2016, Eugene, OR, USA
Affiliation at the time of the award: University of California, San Diego, CA, USA, Howard Hughes Medical Institute
Prize motivation: "for the **development GFP to many kinds of fluorescent protein**"

2008 Nobel Prize in Chemistry

錢永健（Roger Yonchien Tsien，1952年2月1日－2016年8月24日），美國第一代華裔，為美國生物化學家，台灣中央研究院院士、美國國家科學院院士、美國國家醫學院院士、美國藝術與科學院院士；聖迭戈加利福尼亞大學生物化學及化學系教授。因為發現和研究綠色螢光蛋白而獲得了2008年的諾貝爾化學獎。



Nobel Lectures

R. Y. Tsien

DOI: 10.1002/anie.200901916

Green Fluorescent Protein

Constructing and Exploiting the Fluorescent Protein Paintbox (Nobel Lecture)**

*Roger Y. Tsien**

fluorescent proteins · in vivo imaging · mutagenesis · Nobel Lecture

Figure 2. The jellyfish *Aequorea victoria* (or *Aequorea aequorea*) from which aequorin and green fluorescent protein were isolated. Photo courtesy of Dr. Claudia E. Mills, Friday Harbor Laboratories.

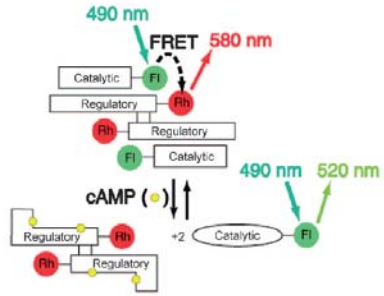


Figure 1. Schematic representation showing how cAMP-induced dissociation of regulatory from catalytic subunits of protein kinase A (PKA) can be reported by loss of FRET from fluorescein to rhodamine labels. Picture: S. Adams, Y. Buechler, S. Taylor.

Prof. R. Y. Tsien
Howard Hughes Medical Ins
Departments of Pharmacology and Chemistry & Biochemistry
University of California, San Diego
9500 Gilman Drive, La Jolla, CA 92093-0647 (USA)
E-mail: rtsien@ucsd.edu

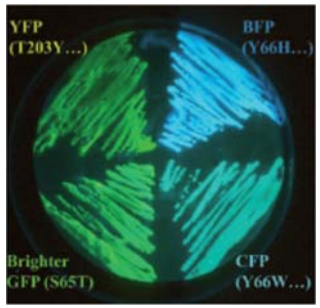


Figure 4. Fluorescence from streaks of E. coli bacteria expressing(clockwise from upper right) improved blue, cyan, green, and yellow fluorescent proteins. Each streak is labeled with the mutation most responsible for its spectral alteration.[25]

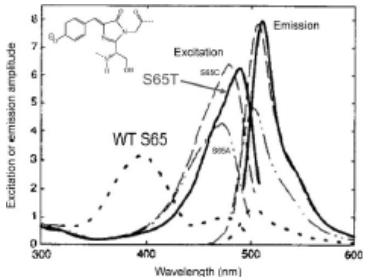


Figure 6. Excitation and emission spectra of wild-type (WT) GFP and several mutants of Ser 65.[25] The inset shows the structure of the wild-type chromophore.

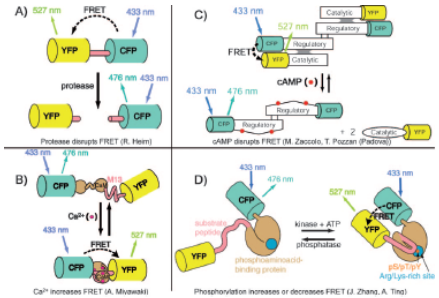


Figure 7. Schematic representations of genetically encoded fluorescent indicators based on FRET from CFP to YFP, to report

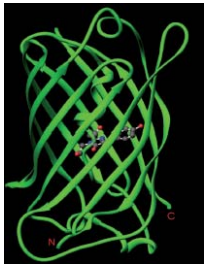


Figure 8. X-ray crystal structure of the S65T mutant of GFP.[31] a Heli-ces and b strands are shown as ribbons, connecting segments as tubes, and the chromophore in ball-and-stick representation. N and C termini are marked.

Figure 11. Chromophore structure[52] of DsRed, drawn on a petri dish using bacteria (E. coli) expressing the protein and showing its beautiful red fluorescence. Structure determination by Larry Gross, drawn by Varda Lev-Ram and Geoff Baird.

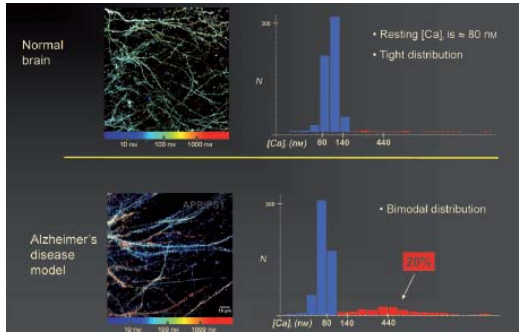


Figure 14. Two-photon imaging of yellow cameleon 3.60[77] in brains of transgenic mice, without or with APP1/presenilin mutations to generate plaques modeling Alzheimer's disease.

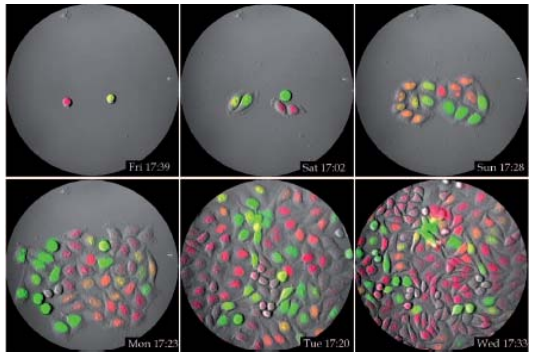


Figure 16. Frames chosen from a time-lapse video of HEK293 cells growing and dividing in tissue culture while transfected with a two-color reporter (YFP and mCherry) of cell-cycle progression. Green-fluorescing cells are actively undergoing mitosis, whereas red-fluorescing cells arein interphase.[62] Video courtesy of Asako Sawano and Atsushi Miyawaki, RIKEN.

Figure 18. High school science classes using fluorescent proteins as laboratory exercises as part of the BioBridge program(biobridge.ucsd.edu).

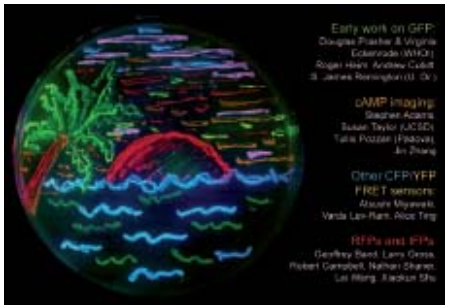
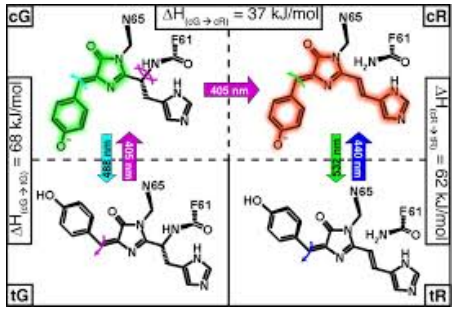


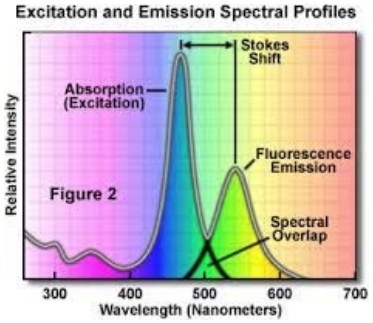
Figure 21. Key collaborators for the work described in this lecture. The glowing California sunset shown in a petri dish on the left was drawn with multiple colors of bacteria expressing fluorescent proteins.

[80] Bright far-red fluorescent protein for whole-body imaging": D.Shcherbo, E. M. Merzlyak, T. V. Chepurnykh, A. F. Fradkov, G. V. Ermakova, E. A. Solovieva, K. A. Lukyanov, E. A. Bogdanova, A. G. Zaraisky, S. Lukyanov, D. M. Chudakov, Nat. Methods 2007, 4, 741 – 746.

PROPERTIES OF GFP

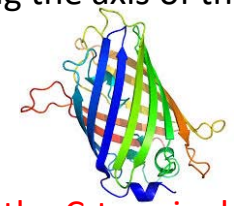
- The native GFP contains 238 amino acids with residues 65-67 (Ser-Tyr-Gly) which is similar to chromophore.





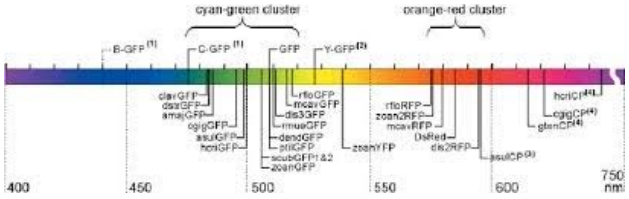
STRUCTURE OF GFP

- GFP is an 11 stranded beta barrel, threaded by an alpha-helix, running up along the axis of the cylinder.



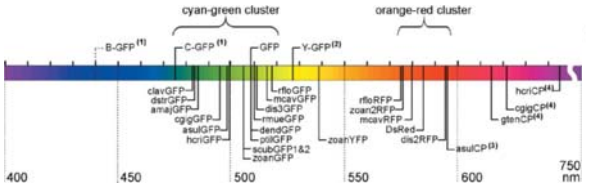
- The N-terminal residue and the C-terminal residues 230-238, approximately corresponding to the maximal numbers of residues than can be removed from the N- (2 residues) and C- (6 residues) respectively of GFP at retained fluorescene.

GFP FAMILY AND ITS MUTANTS

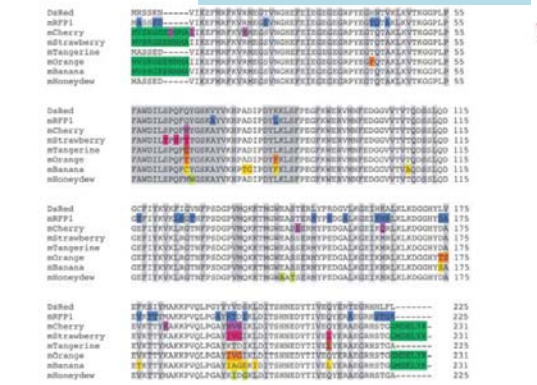


The scientific impact of GFP is it can use in fluorescence correlation spectroscopy (FCS), fluorescence recovery after photo-bleaching (FRAP), photo-activation localization microscopy (PALM)….

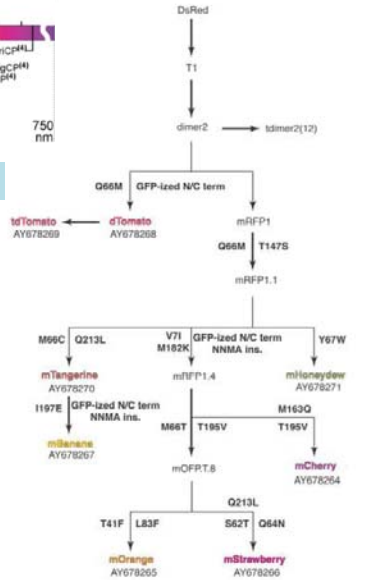
From GFP to RFP derivation



Spectrum and protein alignment



The family of DsRed protein



The spectroscopic and relevant properties derived from DsRed

Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[l]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	26*	25	4.9	Weak dimer[j]

[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 2**. [c]Major excitation peak. [d]Major emission peak. [e]Product of extinction coefficient and quantum yield at pH 7.4 measured or confirmed (indicated by *) in our laboratory under ideal maturation conditions, in (mM • cm)−1 (for comparison, free fluorescein at pH 7.4 has a brightness of about 69 (mM • cm)−1). [f]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH 8.4 has $t_{1/2}$ of 5.2 s); data are not indicative of photostability under focused laser illumination. [g]Brightest in spectral class. [h]Not recommended (dim with poor folding at 37 °C). [i]Citrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. [j]Can be made monomeric with A206K mutation. [k]Emerald has a pronounced fast bleaching component that leads to a very short time to 50% bleach. Its photostability after the initial few seconds, however, is comparable to that of EGFP. [l]Formerly sold by Clontech, no longer commercially available. [m]CFP with A206K mutation; spectroscopic properties equivalent to ECFP.

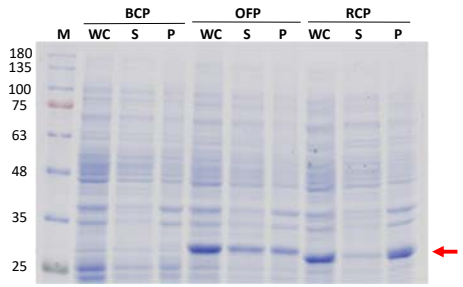
HOW TO EXPRESS GFP related protein in other organisms?

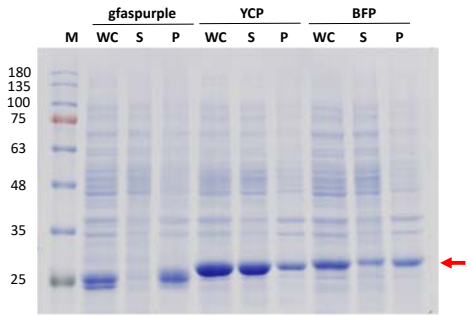
1. Find out the codon optimized GFP gene
2. Construction the expression vector for different organisms. (include promotor, 5'-UTR, 3'-UTR, homologous or non-homologous genetic tool)
3. Transformation to the cell (usually use electroporation! Or Gene gun in multi-chromosomes)

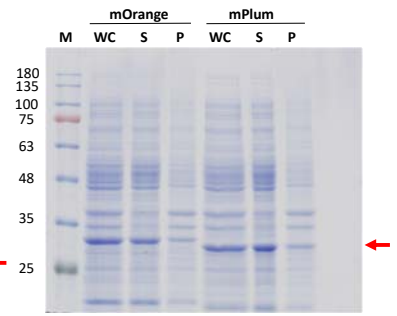
SDS-PAGE Analysis

Condition :

Cell OD_{600nm} = 5
10% gel
S1 : 80V , 30min
S2 : 120V , 70min



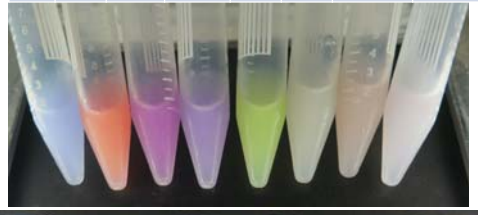




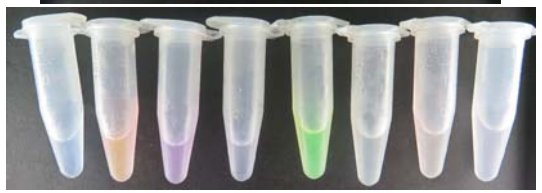
Photo

From left to right :

1	2	3	4	5	6	7	8
BCP	OFP	RCP	gfas purple	YCP	BFP	mOrange	mPlum



Whole cell



Supernatant

Think more: How many cases related on “enzymes” award the “Nobel prize”?

- 英國科學家弗雷德里克·桑格 (Sanger) 1918年8月13日－2013年11月19日
  - 1958年化學獎：測定胰島素分子的結構
  - 1980年化學獎：核酸DNA序列的確定方法
- Hermann Emil Fischer (1852年10月9日－1919年7月15日)在1902年，他因對糖和嘌呤的合成被授予諾貝爾化學獎
  - 1883年他接受巴登苯胺蘇打廠（巴斯夫股份公司的前身）的邀請，前往擔任其實驗室負責人。期間他開始了對糖類的研究。1880年以前，人們已經測出葡萄糖的化學式是C6H12O6，並通過葡萄糖可以發生銀鏡反應和裴林反應推測葡萄糖中存在醛基。

Think more

- 1972 Nobel chemistry prize: 克里斯蒂安·伯默爾·安芬森（Christian Boehmer Anfinsen，1916年3月26日－1995年5月14日）和斯坦福·摩爾與威廉·霍華德·斯坦
  - 一起研究核糖核酸酶，特別是胺基酸序列與生物活性構象之間的關聯
- 1993年凱利·穆利斯、米高·史密斯
  - 因發明聚合酶鏈鎖反應 (PCR)

Think more

- 2002 Nobel chemistry prize: 庫爾特·維特里希、約翰·貝內特·芬恩、田中耕一
  - 開發出的對生物大分子的鑑定和結構分析的核磁共振方法的研究 (即Proteomics)
- 2008 Nobel Chemistry Prize

  下村脩、馬丁·查爾菲、錢永健
  - GFP exploration and application

Think more

- 1933 Nobel Medicine Prize 托馬斯·亨特·摩爾根
  - 他繼承和發展了孟德爾以豌豆雜交實驗為基礎的遺傳理論，同時藉助物理、化學、輻射等實驗手段，為生物學發展為實驗科學奠定了基礎。
  - 他發現位於同一染色體上的基因之間的鏈鎖遺傳特性，將多種突變基因定位在染色體上，製成染色體圖譜，即基因的連鎖圖。
- 1945 Nobel Medicine Prize 亞歷山大·弗萊明、恩斯特·伯利斯·柴恩、霍華德·弗洛里
  - 1928年發現盤尼西林（又名青黴素），這一發現開創了抗生素領域，使他聞名於世。1945年，他與弗洛里和錢恩因為對青黴素的研究活動獲諾貝爾醫學獎。

Think more

- 1952 Nobel Medicine Prize 頒發給瓦克斯曼
  - 自學生時代起研究土壤細菌學, 致力於從各種細菌中篩選分離殺菌成分。因後來他發現了鏈黴素和其他抗生素，被稱為「土壤之人」。
- 1962 Nobel Medicine Prize弗朗西斯·克里克、詹姆斯·杜威·沃森、莫里斯·威爾金斯
  - 發現了脫氧核糖核酸（DNA）的雙螺旋結構。

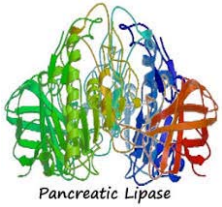
Think more

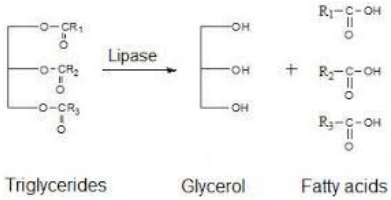

- 1978 Nobel Medicine Prize 頒發給沃納·亞伯、丹尼爾·那森斯、漢彌爾頓·史密斯 (創立冷泉港)
  - 因限制酶的發現，以及關於重組DNA技術的發展研究
- 2009 Nobel Chemical Prize
  - 對核糖體結構和功能的研究而與文卡特拉曼·拉馬克里希南和阿達·約納特共同獲得

Exercise

- How to obtain a mutant of GFP?
  - Hints:
    - Start from a well-known protein
    - Design your steps
- How to analysis the signal of GFP from different cell?
  - Hints:
    - Make sure the excitation and emission wavelength.
    - Consider the instrument you should use

Introduction of Lipase


Pancreatic Lipase



Hydrolysis of triglycerides

OUTLINE

1. What's lipase?
2. How to obtain lipase?
3. The mechanism of lipase and esterase
4. The application of lipase

Lipase introduction

- Esterases (EC 3.1) are a subclass of enzymes that catalyze the hydrolysis of esters to carboxylic acids and alcohols. Carboxylic ester hydrolases (EC 3.1.1)
- A subgroup of esterases to which lipases (triacylglycerol acyl-hydrolases; EC 3.1.1.3) belong (http://www.chem.qmul.ac.uk/iubmb/enzyme/).
- The term lipase can be somewhat misleading since in a very broad sense lipases are enzymes that hydrolyze lipids, which are amply defined in physicochemical terms as fat-soluble molecules.
- More strictly, lipases are considered as enzymes that catalyze the hydrolysis of long chain fatty acids from acyl-glycerols, usually performing at oil–water interfaces

Commercial lipase from Sigma

SIGMA-ALDRICH

Product Comparison Guide

Lipase from *Candida rugosa* 5 Products

Synonym: CCL

CAS Number: 9001-62-1 EC Number: 232-619-9

Enzyme Commission (EC) Number: 3.1.1.3 (BRENDA | IUBMB)

MDL number MFCD00131509

Available Attributes

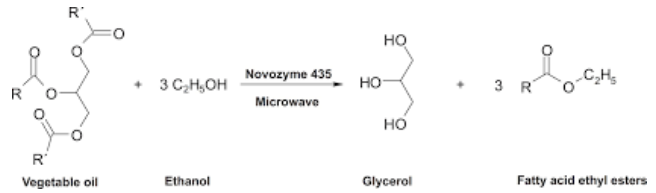
Use the product Attributes below to configure the comparison table. (Select up to 3 total)

Brand | Physical Form | Color

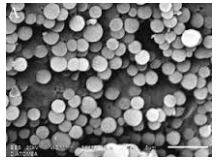
Product #	Description
L1754	Lipase from *Candida rugosa*, Type VII, ≥700 unit/mg solid,

SKU-Pack Size	Availability	Price (USD)	Quantity
L1754-5G	Available to ship on 20.09.16 - FROM	47.60	0
L1754-10G	Available to ship on 20.09.16 - FROM	65.50	0

Commercial lipase from Novozyme® Novo-435



Vegetable oil | Ethanol | Glycerol | Fatty acid ethyl esters



High selectivity, high solvent tolerance, thermostability and fast reaction

Research lipase from *Candida rugosa (CRL)*

Available online at www.sciencedirect.com
SCIENCE DIRECT
BIOTECHNOLOGY ADVANCES
ELSEVIER
Biotechnology Advances 24 (2006) 180–196
www.elsevier.com/locate/biotechadv

Research review paper

Understanding *Candida rugosa* lipases: An overview

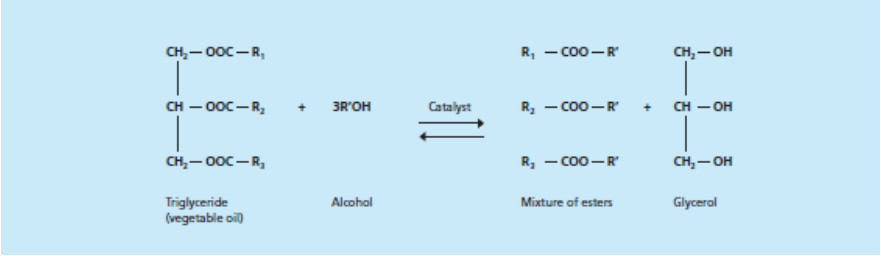
Pablo Domínguez de María, Jose M. Sánchez-Montero,
José V. Sinisterra, Andrés R. Alcántara *

Biotransformations Group, Organic and Pharmaceutical Chemistry Department, Faculty of Pharmacy, Complutense University, Pza. Ramón y Cajal s/n. E-28040, Madrid, Spain

Accepted 26 September 2005
Available online 8 November 2005

Trans-esterification of triglycerides by lipase

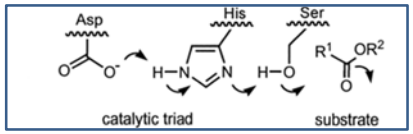
1. Selectivity (Enantioselective ratio, E)
2. Activity ➔ media engineering
3. Stability ➔ immobilization

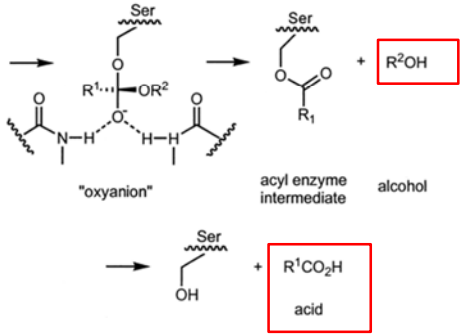


Mechanism

- This mechanism of action can be traced back to some particular structural features that will be described below. The definition of lipases as hydrolases of long chain acylglycerols is rather physiological, because lipases can also hydrolyze carboxylic acids from a variety of compounds of different chemical nature.
- More importantly, in low water activity systems lipases can catalyze the reverse reactions of esterification from fatty acids and glycerol (and other alcohols as well) inter-esterification from two esters or from carboxylic acids and glycerol (and other alcohols as well), and transesterification from an ester and an alcohol. This intended definition of lipases highlights their technological potential

Active site and mechanism





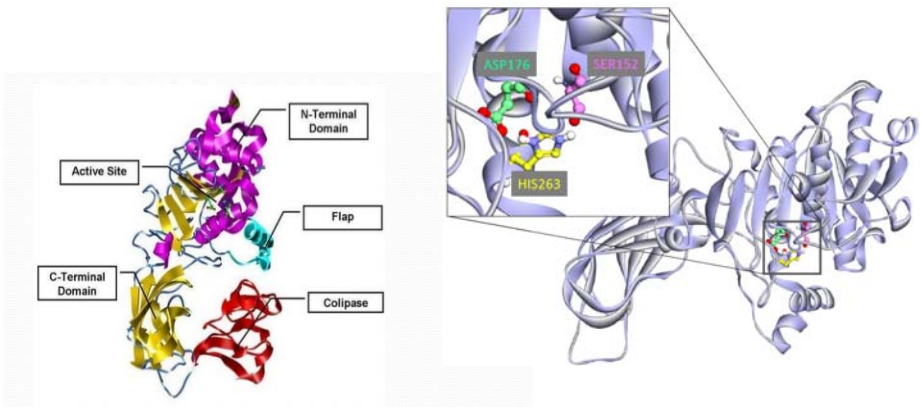
Sources of lipases

- Lipases are widely distributed in nature, being synthesized by plants, animals and microorganisms. Lipases from microorganisms, mainly bacterial and fungal, are the most used as biocatalysts in biotechnological applications and organic chemistry.
- Fungal lipases from *Candida rugosa, Candida antarctica, Thermomyces lanuginosus and Rhizomucor miehei* and bacterial lipases from *Burkholderia cepacia, Pseudomonas alcaligenes, Pseudomonas mendocina* and *Chromobac- terium viscosum* are examples of commercially available lipases widely used in biotechnology.

Sources of lipases

- Only about 2% of the world's microorganisms have been tested as enzyme sources so they offer a huge biodiversity as lipase sources (Hasan et al. 2006).
- Nowadays, 38 distinct bacterial sources of common lipase producers have been referenced (Gupta et al. 2004). In 1998, a search of available data banks revealed 217 entries of lipolytic enzymes from bacteria (Jaeger et al. 1999).

Example: Structure and docking of lipase



Properties of lipases

- lipases have a wide range of properties like positional specificity, enantioselectivity, temperature tolerance and pH dependence (Saxena et al. 2003). Screening and protein engineering techniques are powerful tools for the selection of the most adequate biocatalyst by searching or modulating their catalytic properties (such as substrate specificity or selectivity).
- The extracellular nature of most lipases and recombinant DNA technology make possible to produce them in large quantities by over-expression in an adequate microbial host. Many microbial lipase genes have been cloned, including those of important commercial lipases (Schmidt-Dannert 1999).
- Other lipases from very different sources, like plants (Hong et al. 2000) and extremophilic microorganisms (Demirjian et al. 2001), have been cloned and expressed in *Escherichia coli*.

Properties of different kinds lipase

- Lipase production by *Bacillus* sp. A- 301 required a complex medium that contained different divalent cations such as Ca2+, Mg2+, Co2+, Cu2+, Fe2+, Mn2+, and Mo2+ (Wang et al. 1995). Sharma et al. (2002) observed stimulation in lipase production from *Bacillus* sp. RSJ1in presence of Ca2+, but inhibition by other metal ion salts.
- Other fermentation parameters, such as temperature, pH, agitation and aeration rate are important in microbial lipase production. These parameters are strain dependent: bacteria prefer neutral pHs and the optimum temperature for lipase production corresponds with the growth temperature of the respective organism, which is in the 20–45 °C range, although in some cases optimum temperatures outside that range have been reported Sharma et al. (2002).

Lipase activity and assay

- Lipases may be defined as esterases catalyzing the hydrolysis and synthesis of long-chain fatty acid esters from glycerol. But, there is not a strict definition for "long-chain fatty acid" and, furthermore, most lipases hydrolyze ester substrates (Jaeger et al. 1999) with an acyl chain length of less than 10 carbon atoms with tri-butyrylglycerol (tributyrin) as the standard substrate.
- Lipases hydrolyze triglycerides giving rise to free fatty acids and glycerol. Assays for lipase activity include a wide range of techniques: spectrophotometry, fluorimetry, titrimetry, turbidimetry, surface tension method and estimation of free fatty acids by HPLC (Thompson et al. 1999)

Lipase family

- Lipases have been classified into families. Search of different available data banks (e.g. Swiss Protein Sequence Database) revealed different results based on amino acid sequence homology. Jaeger et al. (1999) identified 47 different bacterial lipases and grouped them into six families.
- Homologous serine hydrolases were assigned to 32 homologous families and 15 super-families by Pleiss et al. (2000). Determination and comparison of specific activities and substrates specificities of different lipases are absolutely required to investigate lipase functions as well as to identify their usefulness for biotechnological applications.
- However, comparison of results is not always possible because no standard substrates are used to determine specific lipase activities or to distinguish lipases from esterases.

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FEMS Microbiology Reviews 26 (2002) 73–81

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Microbial carboxyl esterases: classification, properties and application in biocatalysis

Uwe T. Bornscheuer *

Institute for Chemistry and Biochemistry, Department of Technical Chemistry and Biotechnology, Greifswald University, Soldmannstr. 16, D-17487 Greifswald, Germany

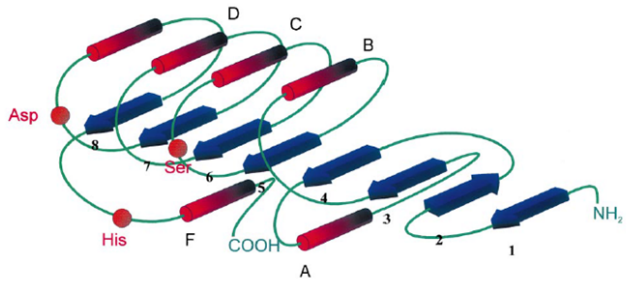


Fig. 1. Schematic presentation of the α/β-hydrolase fold. β-Sheets (1–8) are shown as blue arrows, α-helices (A–F) as red columns. The relative positions of the amino acids of the catalytic triad are indicated as orange circles.

Table 1
Differences between lipases and carboxyl esterases

Property	Lipase	Esterase
Preferred substrates	Triglycerides (long-chain), secondary alcohols	Simple esters, triglycerides (short-chain)
Interfacial activation/lid	Yes	No
Substrate hydrophobicity	High	High to low
Enantioselectivity	Usually high	High to low to zero
Solvent stability	High	High to low

Table 2
Comparison of various (recombinant) microbial carboxyl esterases

Origin[a]	Biochemical properties	Specific substrates[b]
B. gladioli ATCC10248 (EstB)	392 aa, 42 kDa	pNP-esters, triglycerides (up to C_6), deacylates, cephalosporins
B. gladioli ATCC10248 (EstC)	298 aa, 32 kDa	pNP-esters (up to C_5), not triglycerides
P. fluorescens DSM50106	36 kDa, $T_{opt.}$ 43°C	Lactones, ethyl caprylate, moderate enantioselectivity
P. fluorescens SIKW1	27 kDa, homodimer	pNP-esters, high enantioselectivity for α-phenyl ethanol, moderate *E* values for other alcohols and carboxylic acids
Pseudomonas putida MR2068	29 kDa, homodimer, $T_{opt.}$ 70°C	Alkyl-dicarboxylic acid methylesters, high stereoselectivity ($E > 100$)
Bacillus acidocaldarius	34 kDa, $T_{opt.}$ 70°C	pNP-esters (best: hexanoate), moderate stereoselectivity (best: $E \sim 18$)
B. subtilis NRRL B8079	489 aa, 54 kDa, $T_{opt.}$ 52°C (66.5°C for best mutant)	*p*-nitrobenzyl ester of Loracarbef
Bacillus stearothermophilus	–	pNP-esters, moderate enantioselectivity
B. subtilis (ThaiI-8) [c]	32 kDa, $T_{opt.}$ 35–55°C	High enantioselectivity towards 2-arylpropionic acids
Thermoanaerobacterium sp. JW/SL YS485	320 aa, 36 kDa	Xylose tetra acetate, cephalosporin C, MU-Ace[b]
Acinetobacter sp. ADP1	37 kDa	pNP-esters (best: hexanoate), benzyl esters
Clostridium thermocellum	31 kDa	Feruloyl esters
Pyrococcus furiosus DSM3638	$T_{opt.}$ 100°C, $t_{1/2}$ 50 min at 126°C	MU-Ace[b]
Lactococcus lactis[e]	258 aa, 30 kDa	pNP-esters (best: hexanoate), tributyrin, C_6-phospholipids
Rhodococcus ruber DSM 43338[d]	Two esterases with opposite enantiopreference	Linalool-acetate ($E > 100$)

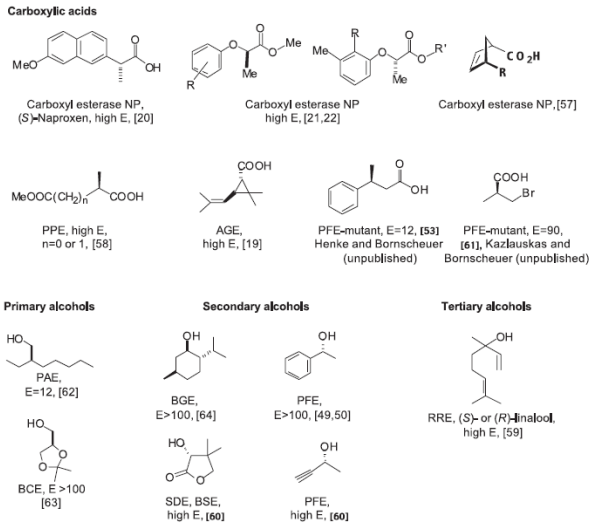
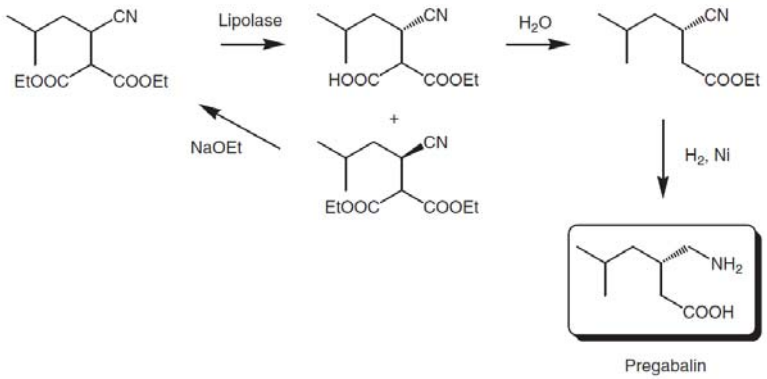


Fig. 2. Selected examples of chiral compounds obtained by carboxyl esterase-catalyzed kinetic resolutions. AGE, esterase from *A. globiformis*; BCE, esterase from *Bacillus coagulans*; BSE, esterase from *Bacillus stearothermophilus*; BGE, esterase from *B. gladioli*; PAE, esterase from *P. aeruginosa*; PME, esterase from *Pseudomonas marginata*; PPE, esterase from *Pseudomonas putida*; RRE, esterase from *Rhodococcus ruber*; SDE, esterase from *Streptomy-*

Lipase based reaction: resolution



This is one of the most important application of lipase!

Lipase vs esterase

(1) Lipase has property of surface activation

(2) Lipase is more complexes.

(3) Esterase always used p-nitrophenyl esters as substrates

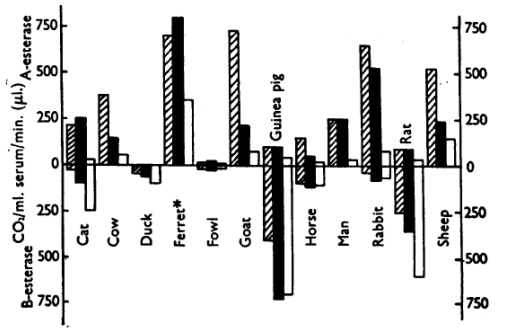


Fig. 7. Histogram of the rates of hydrolysis of *p*-nitrophenyl esters by the sera of various species. Substrates: ▨, *p*-nitrophenyl acetate; ■, propionate; □, butyrate. * For convenience the rates of hydrolysis plotted for ferret serum are one-half the rates actually determined.

Tips: directed evolution for improved enzyme function

1) What's directed evolution?

2) What's E value?

3) How can you do that?

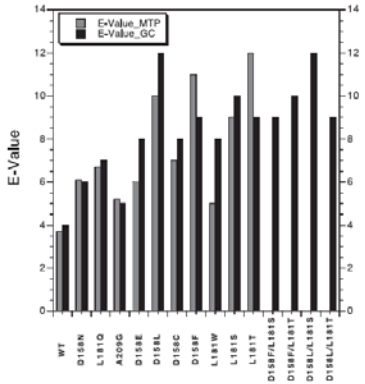


Fig. 3. Mutants obtained by directed evolution and saturation mutagenesis showing enhanced enantioselectivity in the resolution of 3-phenylbutyric acid derivatives. MTP: E values (E_{app}) determined in microtiter plates using the corresponding (*R*)- and (*S*)-resorufin ester [53]; GC: E values (E_{true}) calculated according to literature [56] from data determined by gas chromatographic analysis on a chiral column from samples obtained after esterase-catalyzed hydrolysis of (*R,S*)-3-phenyl butyric acid ethylester [50].

Lipase assay

Method 1: Assay using olive oil as substrate

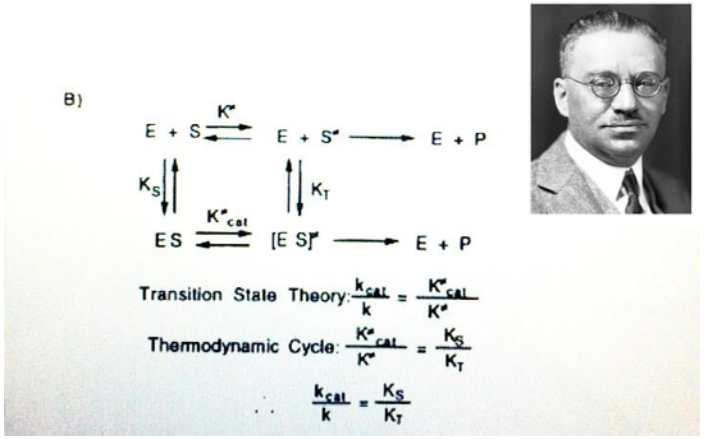
Esterase activity was performed with olive oil, which was prepared as follows: the reaction mixture containing 5 mL of olive oil, 2 mL of 0.1M phosphate buffer (pH 7.0) and 1 mL of the enzymatic extract (10 mg.mL^{-1}) was incubated at 37 °C for 30 minutes with orbital shaking. Immediately after incubation, the system was disrupted by the addition of 15 mL of acetone-ethanol mixture (1:1 v/v) and the liberated free fatty acids were titrated with 0.05M NaOH. All assays were done independently and in duplicate. One unit of esterase activity was defined as the amount of enzyme which liberated 1 µmol of fatty acids per minute.

Esterase assay-2

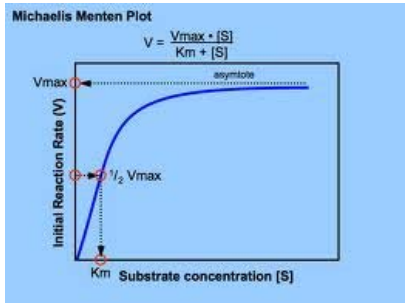
Method 2: Assay using p-nitrophenyl butyrate (pNPB) as substrate

Esterase activity was determined spectrophotometrically following the hydrolysis of *p*-nitrophenylbutyrate (*p*NPB) at 405 nm. An aliquot (0.070 mL) of the enzyme suspension (10 mg.mL^{-1}) was added to 3.43 mL of a reaction mixture with the following composition: 1.12 mM *p*NPB dissolved in 50 mM phosphate buffer, pH at 7.2, also containing 0.2% (N/P) Triton X-100 and 0.43M tetrahydrofuran. The reaction was monitored for 15 minutes against blank solution (CALADO et al., 2002). All assays were carried out independently and in duplicate. One unit of esterase activity was defined as the amount of esterase required to release 1 µmol of *p*-nitrophenol in one minute, under the specified conditions. *p*NPB was purchased from Sigma-Aldrich Brasil (Sao Paulo, Brazil).

Michaelis Menten equation



Michaelis Menten Plot



- A kinetic model established at the constant amount of enzyme, the velocity of the reaction is determined at various substrate concentration.
- Under zero-order condition, velocity is directly dependent on [enzymes].

Michaelis, L.,and Menten, M. (1913) Die kinetik der invertinwirkung, *Biochemistry Zeitung 49*, 333-369.

Briggs, G. E., and Haldane, J. B. (1925) A Note on the Kinetics of Enzyme Action, *Biochem J 19*, 338-339.

Van Slyke, D. D., and Cullen, G. E. (1914) The Mode of Action of Urease and of Enzymes in General, *J Biol Chem 19*, 141-180.

Michaelis Menten Equation at steady state:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[(v_o)]{k_3} E + P$$

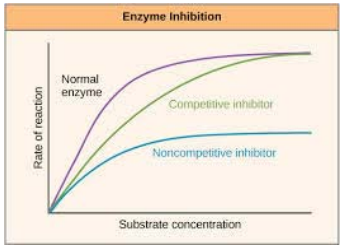
$$k_1[E][S] = k_2[ES] + k_3[ES] \quad (I)$$

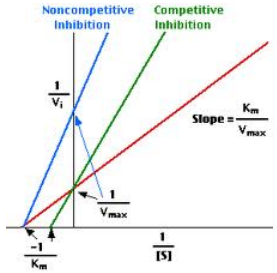
$$[Et] = [Ef] + [ES] \quad (II)$$

$$v_o = k_3[ES] \quad (III)$$

$$V_{max} = k_3[Et] \quad (IV)$$

Lineweaver-Burk plot

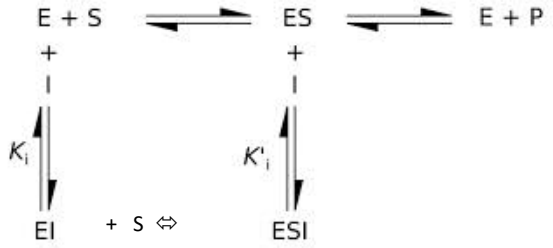




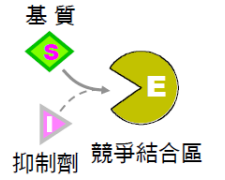
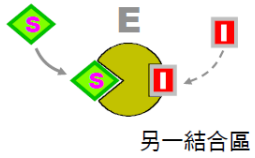
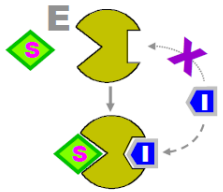
$$V_0 = \frac{Vmax\,[S]}{Km + [S]}$$

Enzymatic inhibition

- E + I ⇔ EI or I + ES ⇔ IES or E + IS ⇔ EIS

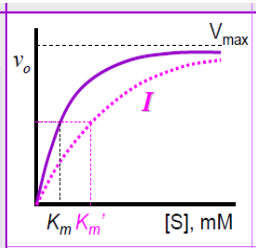
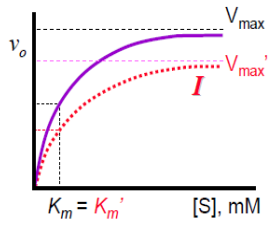
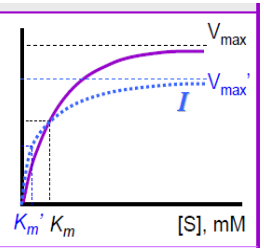
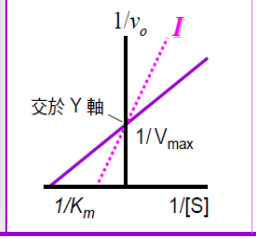
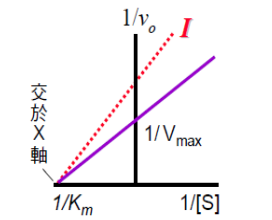
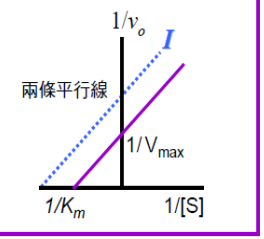


Reference book:
微生物酵素工程學，陳國誠教授編著，藝軒圖書出版社 (1988)
Microbial Enzyme Engineering

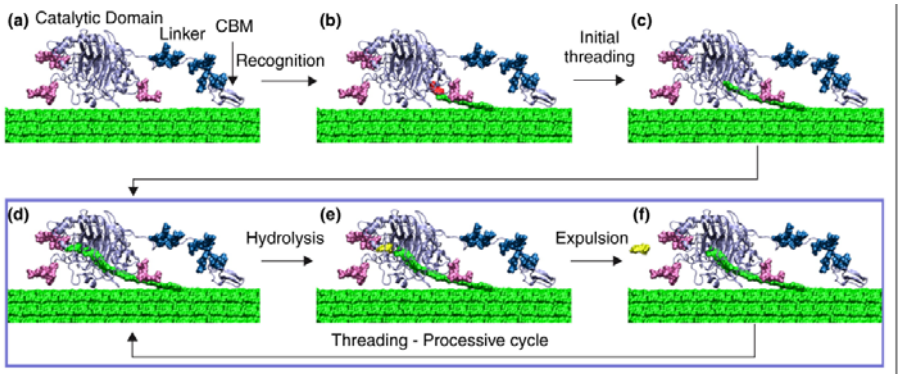
Competitive	Non-competitive	Uncompetitive
		
$E + S \rightleftarrows ES \rightarrow E + P$ + *I* ⇵ *EI*	$E + S \rightleftarrows ES \rightarrow E + P$ + + *I* *I* ⇵ ⇵ *EI* + S → *EIS*	$E + S \rightleftarrows ES \rightarrow E + P$ + *I* ⇵ *EIS*
[*I*] 只與自由的 [E] 結合，會與 [S] 競爭；[S]↑可克服 [*I*] 的抑制。	[*I*] 可與自由的 [E] 或已佔據有 [S] 的 [ES] 結合，[S]↑不能克服 [*I*] 的抑制。	[*I*] 只能與 [ES] 結合，[S]↑反而有利 [*I*] 的抑制。

台灣大學莊榮輝教授
http://juang.bst.ntu.edu.tw/

Different kinds of inhibition

Direct graph			
	V_{max} 不變；K_m 變大	V_{max} 變小；K_m 不變	V_{max} 及 K_m 均變小
Reciprocal plot			

Enzyme used in Biofuel: Cellulase system



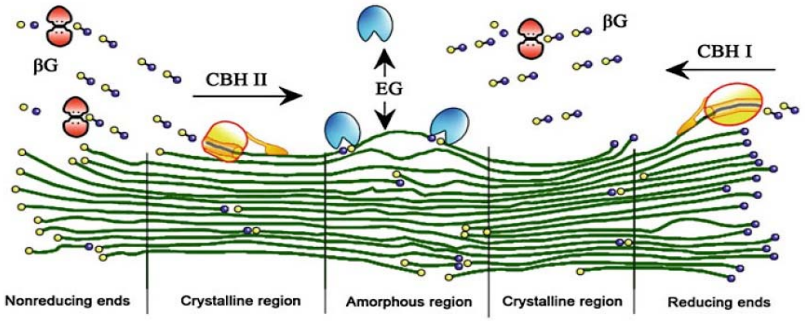
Outline

- Introduction and sources
- Mechanism
- Enzyme assays
- Application and challenges

What's cellulase?

• Cellulase is an enzymatic complex composed usually by an exo acting hydrolase (1,4 β-D-glucan cellobiohydrolase) an endo acting hydrolase (1,4 β-D-glucan glucano hydrolase) and a cellobiase (β-D-glucoside glucohydrolase, beta-glucosidase).

• These fractions act synergistically to breakdown the cellulose fibers down to glucose. Cellulases have many and increasing applications in the food, feed, detergent and textile industries



How to use cellulase in biofuel?

- It is estimated that only lignocellulose derived ethanol can meet this challenge, so considerable effort is now being spent to overcome the technological limitations still prevailing (Wyman 1996; Sheehan and Himmel 1999). Among those, the requirement of more active and more stable cellulases is crucial.
- The goal is to reduce the cost of using cellulase enzymes by front line technology with an expected reduction from about US$ 0.1 to about US$ 0.015 per liter of bioethanol. This requires significant increase in specific activity and production efficiency. Optimized combinations of the different cellulase fractions have been successfully and promising results have been obtained in cellulase improvement by genetic and protein engineering techniques.

Hemicellulase and Ligninases

• Enzymes have very relevant applications in the pulp and paper industry now threatened by environmental regulations.

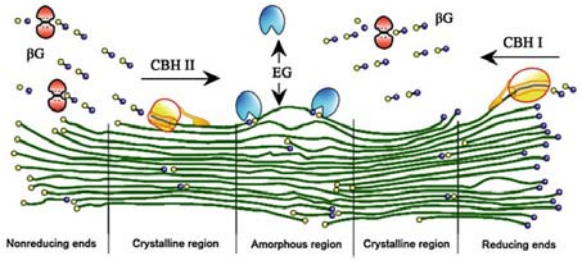
• Hemicellulases are being currently used in wood pulp bleaching to partially substitute chlorine-based bleaching procedures that produce harmful chlorinated organic compounds and an increasing market for this enzyme has developed in the last decade.

• Ligninases have a great potential both in wood bleaching and pulping; however, ligninases are quite complex coenzyme requiring enzymes being this complexity a major hurdle for its massive application.

Trichoderma reesei: Most powerful fungus in degradation of cellulose

The cellulose biodegradation by cellulases produced by numerous microorganisms has been widely employed for producing sustainable bio-based products and biofuels to replace the depleting fossil fuels [6, 7]; however, currently the utilization of cellulosic biomass for bioethanol faces significant technical and economic challenges. Its success largely depends on the development of highly efficient and cost-effective biocatalysts for the conversion of pretreated biomass to fermentable sugars. In the past several decades, many studies focus on *Hyprocreaceae* (*Trichoderma*), a filamentous fungus present in nearly all soils and plant roots and having the capability to secrete large amounts of cellulolytic enzymes [8–10]. Therefore, cellulases produced by fungi, especially by *T. reesei*, have been extensively studied. In spite of the present success of such work, the task of finding new highly active cellulases or efficient producers of cellulases from various fungi with intact lignocellulosic enzymes secreting to the medium remains an active concern.

Key enzymes for 2nd generation of biofuel: cellulases

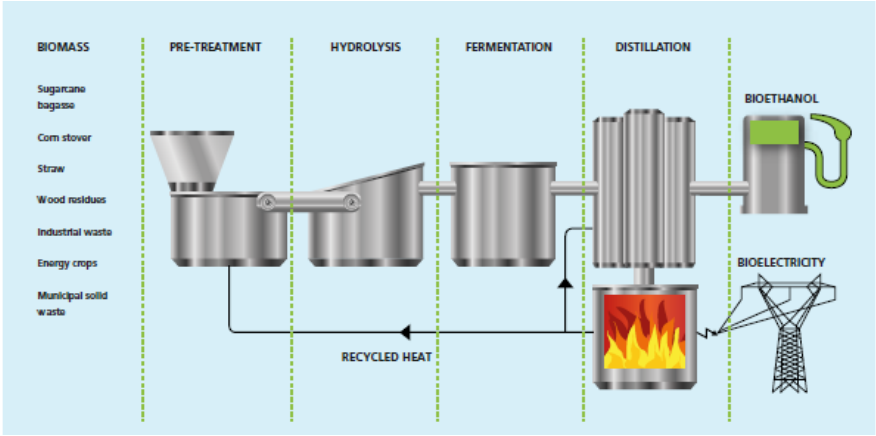


- The celluloses are rigid and strong structure with two regions: amorphous and crystalline.
- Cellulase system are including endo-glucanase, exo-glucanase and beta-glucosidase , which work together to degrade the cellulose to glucose absolutely.

Qu et al. Adv Biochem Engin Biotechnol (2012)

General process for 2nd generation biofuel: ethanol production

1. Pretreatment (need enzymes)
2. Hydrolysis (need enzymes)
3. Fermentation (need enzymes)
4. Distillation



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Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production

Rajeev K. Sukumaran, Reeta Rani Singhania, Gincy Marina Mathew, Ashok Pandey*

Biotechnology Division, National Institute for Interdisciplinary Science and Technology (formerly Regional Research Laboratory), CSIR, Trivandrum-695 019, India

Table 1
Testing of saccharification conditions for hydrolysis of sugar cane bagasse

Method	Cellulase loading (FPU/g)	BGL loading (U/g)	Incubation time (h)	Reducing sugar yield g/L	Endoglucanase activity (CMCase assay)		BGL activity (pNPG assay)	
					U/gDS	U/ml	U/gDS	U/ml
A	80	10	24	3.79	299.55	14.98	4.5	0.22
B	50	5	48	17.64	NA	45.22	NA	1.72
					135.44	6.77	21.39	2.84
					NA	196.15	NA	83.62

Table 2
Comparison of the crude enzyme preparations from *T. reesei* RUT C30 and *A. niger* MTCC 7956

Fungus	Type of enzyme preparation	Total cellulase activity (Filter paper assay)	
		FPU/gDS	FPU/ml
Trichoderma reesei RUT C30	Crude cellulase (SSF extract)	22.8	1.14
	Concentrated Cellulase (10×)[a]	NA	13.65
Aspergillus niger MTCC 7956	Crude BGL (SSF extract)	4.55[b] (U/gDS)	0.22[b](U/ml)
	Concentrated BGL (30×)[a]	NA	0.24

From cellulase to ethanol

Table 4
Ethanol production from rice straw hydrolysate by *Saccharomyces cerevisiae*

Reducing sugar (RS) concentration (g/L)	Duration of incubation (h)	Ethanol concentration (g/L)	Ethanol yield (g/g DS)	Efficiency (RS to ethanol) %
60	24	12.34	0.093	40.33
120	24	25.56	0.096	41.76

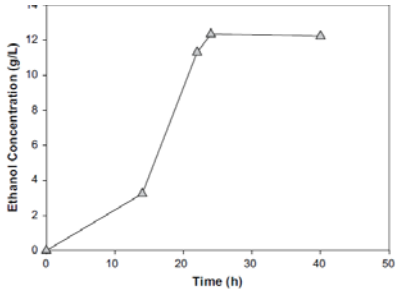


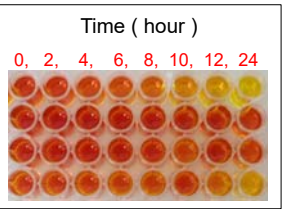
Fig. 1. Time course of ethanol production by *Saccharomyces cerevisiae* MTCC 7956 from alkali pretreated rice straw hydrolysate (6% initial reducing sugar concentration).

Reducing sugar and activity

samples Time (hour)

0, 2, 4, 6, 8, 10, 12, 24

MRS
Glucose
Lactose
Sucrose



Activity:

Δ (OD) / slope of DNS calibration curve = mg/mL = sugar content

Unit = sugar content
*0.2/180*1000/time (min)

DNA sequences and expression in Streptomyces lividans of an exoglucanase gene and an endoglucanase gene from Thermomonospora fusca.

E D Jung, G Lao, D Irwin, B K Barr, A Benjamin and D B Wilson
Appl. Environ. Microbiol. 1993, 59(9):3032.

TABLE 3. CMC activities of E1 and E4 in *E. coli* and *S. lividans* cultures

Enzyme, strain, and plasmid(s)	Assay	Assay data: Amt assayed (μl)	Assay data: Time (min)	Assay data: Reducing sugar produced (μmol of CB*)	Culture activity (μmol of CB/min/liter)
E1					
E. coli pSHE1	10× extract	20	30	0.763	130
	Supernatant	100	1,320	0.134	1.0
S. lividans pSHE1	Supernatant	10	30	0.575	1,920 ± 190
E4					
E. coli					
pSE4-1	10× extract	100	1,320	0.263	0.20
	Supernatant	100	1,320	0.006	0.05
pSE4-2	10× extract	10	960	0.471	4.9
	Supernatant	100	1,320	0.193	1.5
pSE4-1A	10× extract	100	1,320	0.143	0.11
	Supernatant	100	1,320	0.032	0.24
S. lividans					
pSE4-1	Super	20	960	0.440	22.9 ± 0.7
pSE4-2	Super	20	960	0.395	20.6 ± 0.2
pSE4-1A	Super	20	960	0.314	16.4 ± 0.6

* CB, cellobiose.
* Based on CMC-specific activities of 53.6 and 1.36 μmol of CB/min/mg for E1 and E4, respectively (21).

DNS assay: determine non-reducing sugar

- This method tests for the presence of free carbonyl group (C=O), the so-called reducing sugars. This involves the oxidation of the aldehyde functional group present in, for example, **glucose and the ketone functional group in fructose.** Simultaneously, 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

oxidation

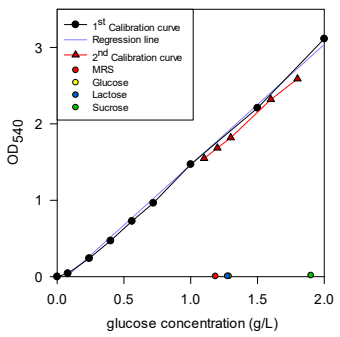
- **aldehyde group ----------> carboxyl group**

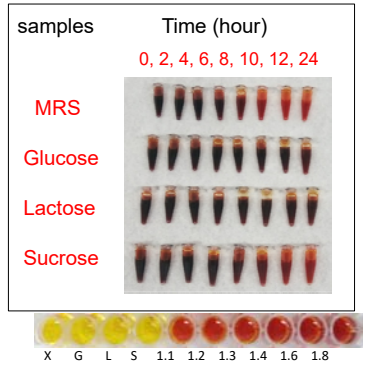
reduction

- **3,5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid**

- Different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reactions such as the decomposition of sugar also competes for the availability of 3,5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color.

DNS calibration curve





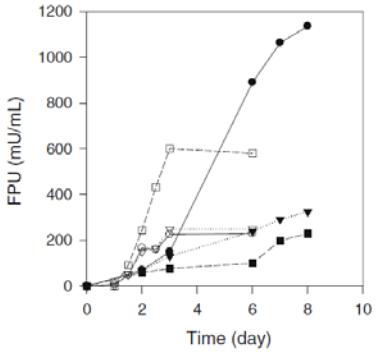
•Dinitrosalicylic Acid Reagent Solution (1%)
- Dinitrosalicylic acid: 10 g
- Sodium sulfite: 0.5 g
- Sodium hydroxide: 10 g
- Add water to: 1 liter

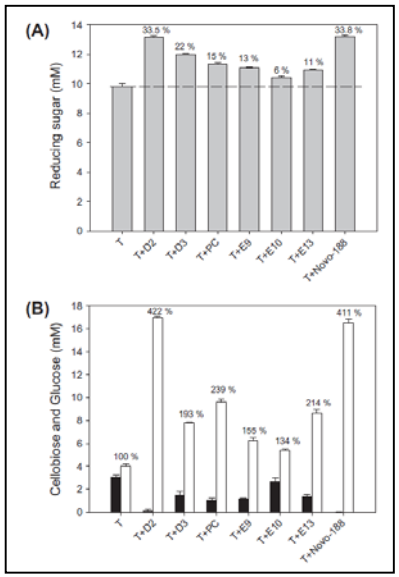
•potassium sodium tartrate solution, 40%

1. Add 0.5 ml of DNS reagent to 0.5 ml of sample
2. Heat the mixture at 100º C for 10 minutes to develop the red-brown color.
3. After cooling to room temperature (or put on ice), record the absorbance with a spectrophotometer at 550 nm.

Different assays

- CMCase
- FPase
- Beta-glucosidase by p-nitro compound
- HPLC (the best approach)





Pioneer in Cellulase study

Lee Rybeck Lynd
Dartmouth College, Thayer School of Engineering, Hanover, USA

Professor *Lynd* is an expert on the production of energy from plant biomass and conducts leading research on microbial cellulose utilization.

The research lab at Dartmouth led by Professor Lee Lynd is active in research on the following topics:

- **Microbial Cellulose Utilization, including fundamental and applied aspects**
- **Metabolic Engineering, focusing on thermophilic cellulolytic bacteria for fuel production**
- **Innovative Biomass Processing Technologies, including development, design, and evaluation**
- **Sustainable Bioenergy Futures, including resource, environment, and economic development**

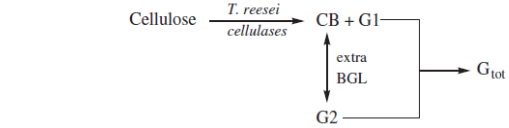
Application and Challenges

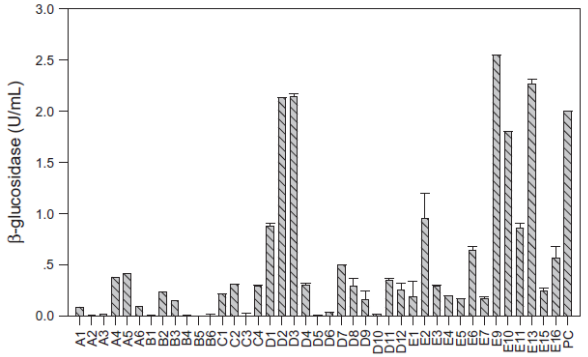
1. What's the critical point in this study?
2. Any new enzymes?
3. How to improve the activity in the system?
4. What's SSF (Spontaneously saccharification fermentation)?

1. Cost effective
2. Explore and direct evolution
3. Synergism effect
4. Super host or in vitro system

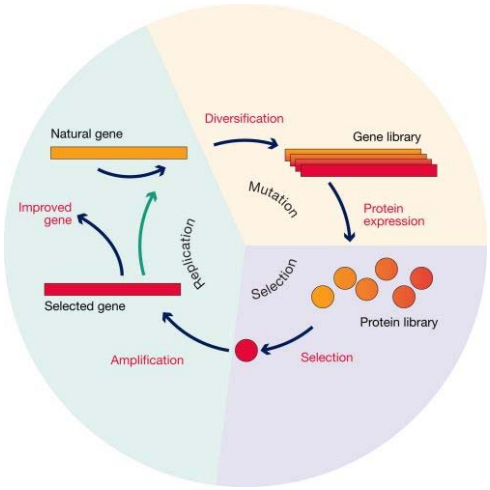
Application and Challenges

[1] How to improve the activity in the system?





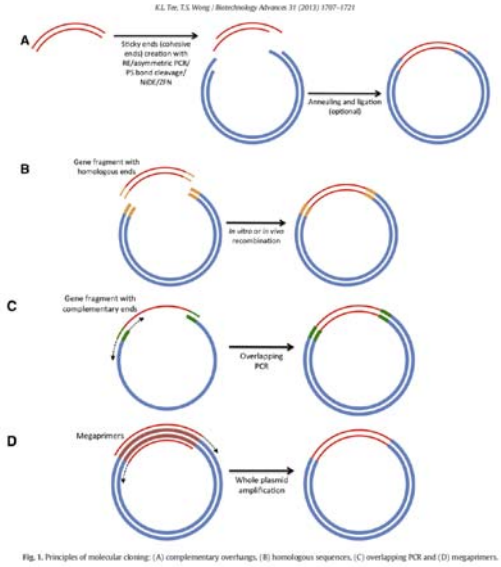
Technology used in Enzyme evolution



Whole gene and site-directed random mutagenesis for improved enzyme property

- Random mutagenesis
  - Error-Prone PCR
  - In vitro gene recombination: chimera
  - UV mutation
  - Optimal library design with high-throughput screening
  - DNA shuffling

Error-prone PCR



(1) Polymerase
(2) $MnCl_2$
(3) A/T/G/C ratio

DNA shuffling

- DNA shuffling is a way to rapidly propagate beneficial mutations in a directed evolution experiment. It is used to rapidly increase DNA library size, because it is a recombination between different DNA species with different mutations.

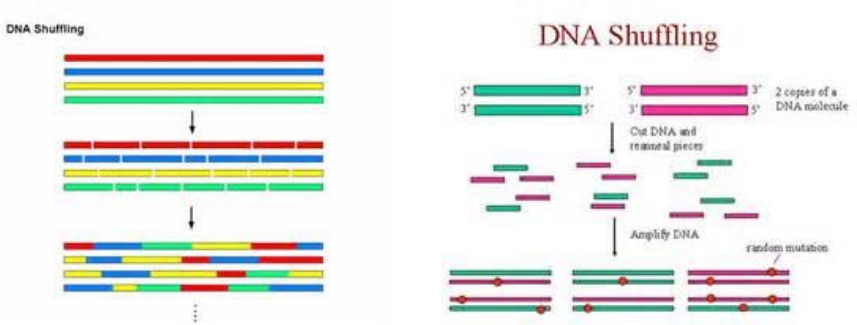
Procedure

- DNase is used to fragment a set of parent genes into pieces of 50-100 bp in length.
- This is then followed by a polymerase chain reaction (PCR) without primers- DNA fragments with sufficient overlapping homologous sequence will anneal to each other and are then extended by DNA polymerase.

Over extension by PCR

- Several rounds of this PCR extension are allowed to occur, after some of the DNA molecules reach the size of the parental genes.
- These genes can then be amplified with another PCR, this time with the addition of primers that are designed to complement the ends of the strands.
- The primers may have additional sequences added to their 5' ends, such as sequences for restriction enzyme recognition sites needed for ligation into a cloning vector.

To have a big library!



Rational design

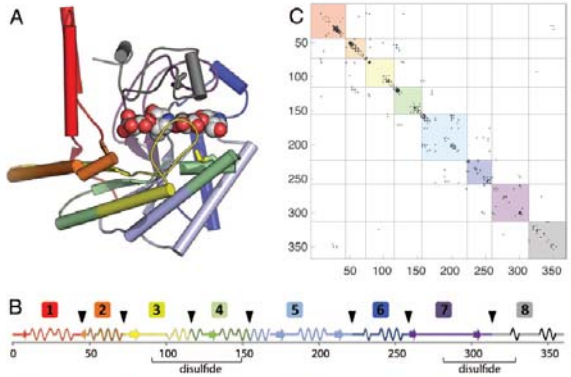


Fig. 2. Illustrations of CBH II chimera library block boundaries. (*A*) *H. insolens* CBH II catalytic domain ribbon diagram with blocks distinguished by color. CBH II enzyme is complexed with cellobio-derived isofagomine glycosidase inhibitor (13). (*B*) Linear representation of *H. insolens* catalytic domain showing secondary structure elements, disulfide bonds and block divisions denoted by black arrows. (*C*) Sidechain contact map denoting contacts (side chain heavy atoms within 4.5Å) that can be broken upon recombination. The majority of broken contacts occur between consecutive blocks.

DNA shuffling pioneer: Frances H. Arnold

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Frances H. Arnold

Dick and Barbara Dickinson Professor of Chemical Engineering, Bioengineering and Biochemistry; Director, Donna and Benjamin M. Rosen Bioengineering Center

B.S., Mechanical and Aerospace Engineering, Princeton University, 1979; Ph.D., Chemical Engineering, University of California, Berkeley, 1985; Postdoctoral, UC Berkeley, Chemistry, 1985; Postdoctoral, Caltech, Chemistry, 1986

Office Location: 228B Spalding

Available online at www.sciencedirect.com
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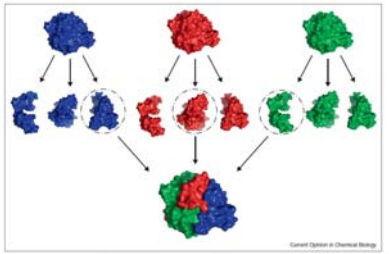
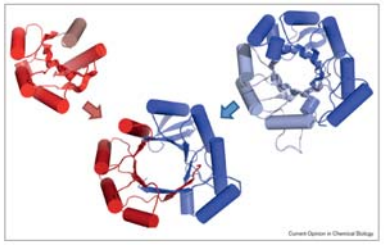
Current Opinion in Chemical Biology

Innovation by homologous recombination

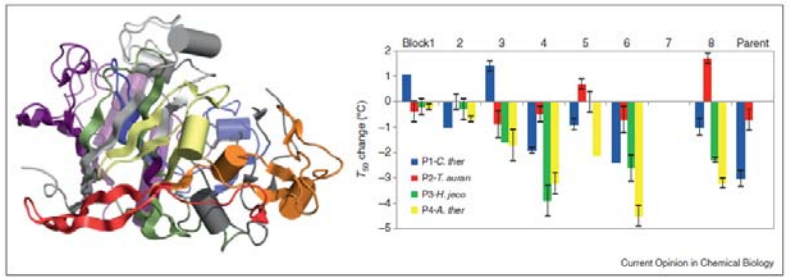
Devin L Trudeau, Matthew A Smith and Frances H Arnold





Enhance thermo-stability

Figure 3



Thermostability contributions of recombined blocks. Heinzelman *et al.* made a chimera library of class I cellobiohydrolases (CBHI), with parent enzymes from *T. emersonii*, *C. thermophilum*, *T. aurantiacus*, *H. jecorina*, and *A. thermophilum*. Recombination sites chosen by SCHEMA generated the blocks shown in different colors on the *T. emersonii* CBHI structure (left). Individual blocks make different contributions to thermostability when introduced into *T. emersonii* CBHI (right). Thermostabilizing blocks were combined to make thermostable chimeras.
Modified from Ref. [14].

http://www.codexis.com/

CODEXIS

Founded in California in 2002, Codexis was established specifically to provide unique enzyme optimization services and biocatalyst products for use in industrial processes across the pharmaceutical sector and related markets.

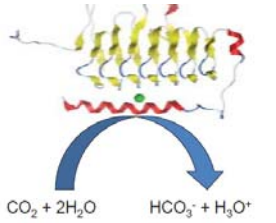
Discover our full range of products, including screening kits, directed evolution services and enzyme production platforms.

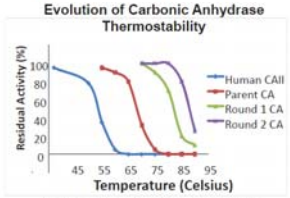
Total revenues of $46 million to $49 million, representing growth of 10% to 17% over 2015.

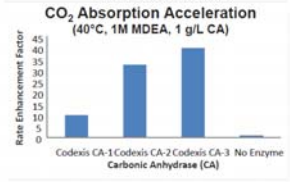
CODEXIS

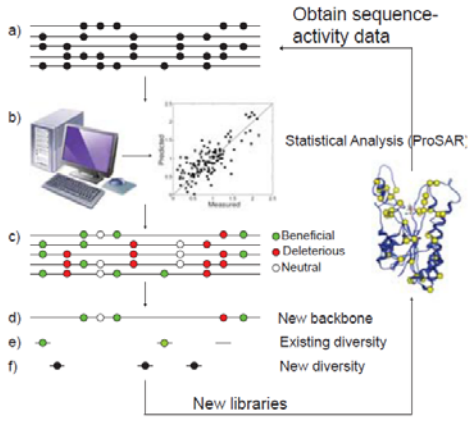
Low-Cost Biocatalyst for Acceleration of Energy Efficient CO_2 Capture

Chris Savile, Luan Nguyen, Oscar Alvizo, Svetlana Balatskaya, Gerald Choi,[1] Michael Benoit, Irene Fusman, Joshua Geilhufe, Sunil Ghose,[1] Svetlana Gitin, John Grate, Jack Liang, Lisa Newman, Scott Novick, Jaime Parsons, James Riggins, Sabrina Zimmerman, and Jim Lalonde
Codexis, Inc., 200 Penobscot Dr., Redwood City, CA 94063, USA
[1] Nasset Inc., San Francisco, CA
jim.lalonde@codexis.com









2016 Tang Prize winner

CRISPR/Cas9 genome editing

What's Tang Prize?

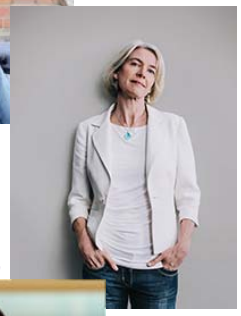
- 唐獎面對當前社會發展，以中華文化數千年的涵養，從新視野與新思維注入實際的行動與思考。有感於全球化的進步與發展，在人類享受文明的豐厚果實與科技所帶來的便利之時，人類亦面臨氣候變遷、貧富差距、社會道德式微等等之考驗。**尹衍樑博士於2012年12月成立唐獎，設置永續發展、生技醫藥、漢學及法治四大獎項**，而且不分種族與國籍，遴選出對世界具有創新實質貢獻及影響力的成就者。
- 唐獎四大獎項領域考究的是二十一世紀人類所需要的智慧，並勉勵時代先驅者以其學說易天下，以天下為己任，共同為世界文明而努力。

What's Tang Prize

- 「永續發展」表彰對人類在地球上永續生存與發展具開創性及卓越貢獻者；「生技醫藥」著重生物醫學或藥物研發，並有效解決人類疾病，提升健康與生活品質之貢獻；「漢學」指其廣義定義，重點在彰顯中華文化，促進人類內在的精神自覺；「法治」之目標則基於人生而平等的信念，期待建立更為普及與完善的制度，藉以實踐人類及自然之共同福祉。
- 唐獎發軔於東方思想的沃土，以其文化價值、精神與世界相互調和，並淬聚人類智慧與全球知識，期待成為二十一世紀永續發展的重要推手，以便為世界之美好貢獻力量，展現新時代的價值與意義。

CRISPR/Cas9 discovery

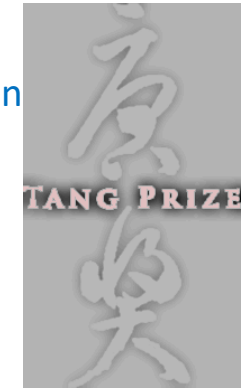
- **Emmanuelle Charpentier**
  Max Planck, Germany
- **Jennifer Doudna**
  UC Berkeley, USA
  http://rna.berkeley.edu/index.html



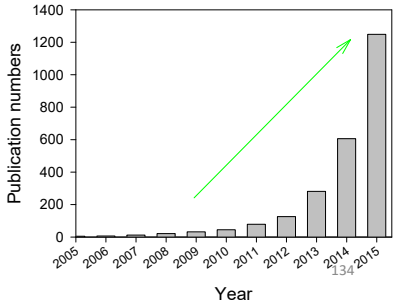
- **Learn Feng Zhang in Broad Institute, MIT, USA**
  http://zlab.mit.edu/index.html

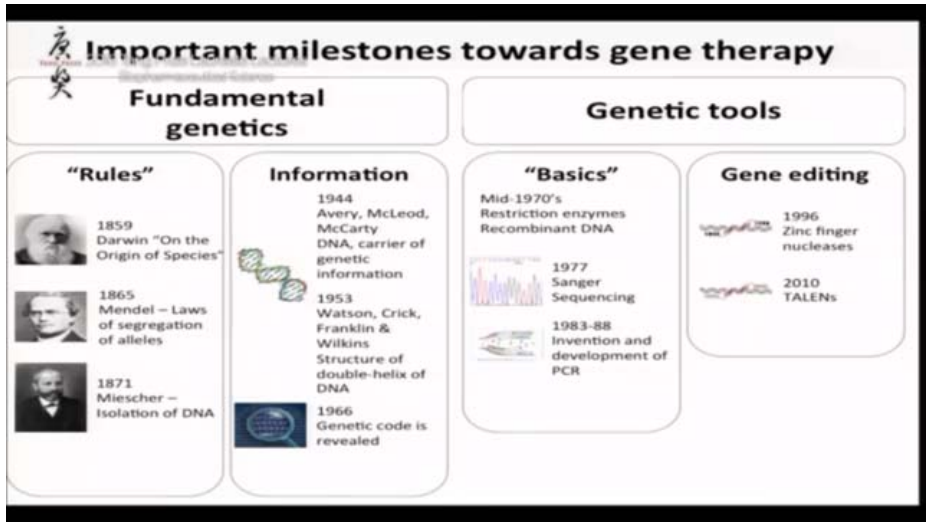
2016 Tang Prize Laureate

Announcement on June 19, 2016, Taiwan



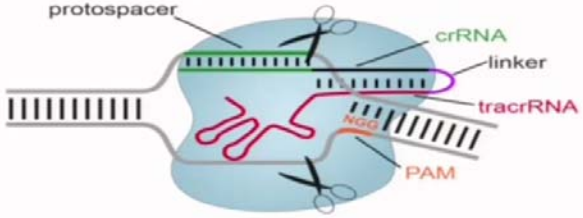
- Biopharmaceutical Science award to
  Dr. Emmanuelle Charpentier,
  Dr. Jennifer A. Doudna and
  Dr. Feng Zhang
- They develop the "CRISPR/Cas9" system which have high impacts on **biopharmaceutical platform** and **gene therapy**.





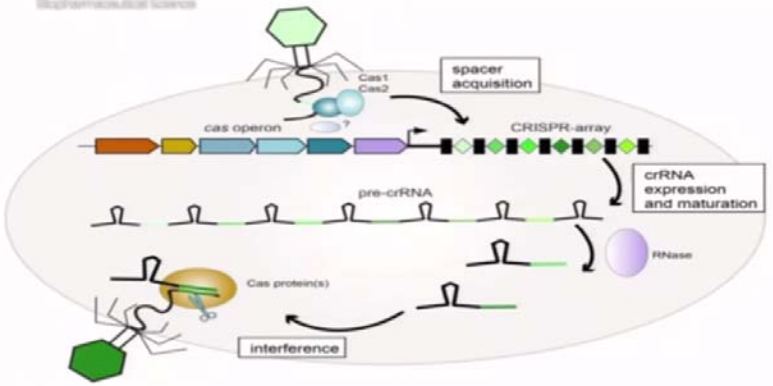
CRISPR-Cas9 technology: a two-component system

The enzyme Cas9 is programmed
with a dual or single guide RNA
to target and cleave specifically DNA



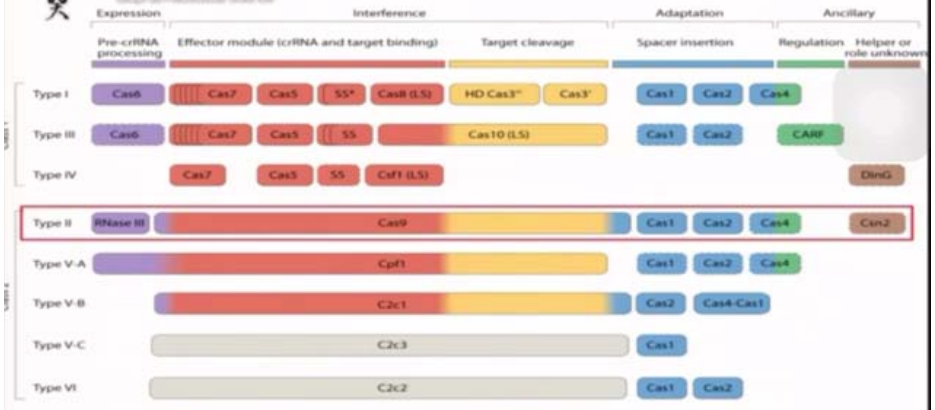
Deltcheva et al., 2011 (Nature); Jinek, Chylinski et al., 2012 (Science)

The CRISPR-Cas adaptive immune system



CRISPR = Clustered regularly interspaced short palindromic repeats

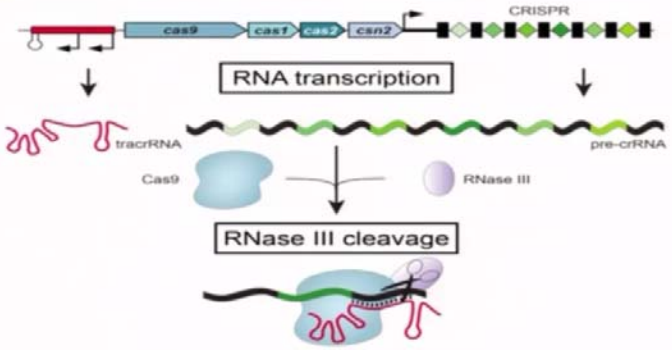
CRISPR-Cas has evolved into six major types



Adopted from Makarova et al. (Nature Rev. Microbiol., 2015)

Type II CRISPR-Cas: model of crRNA maturation

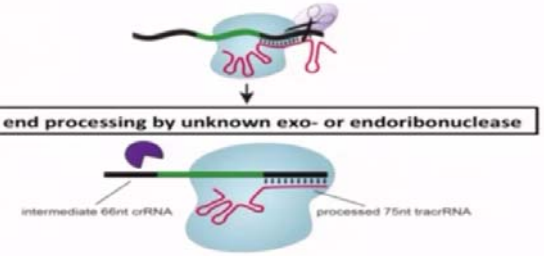
- 1rst processing event: RNase III cleavage



Deltcheva et al. (Nature, 2011)

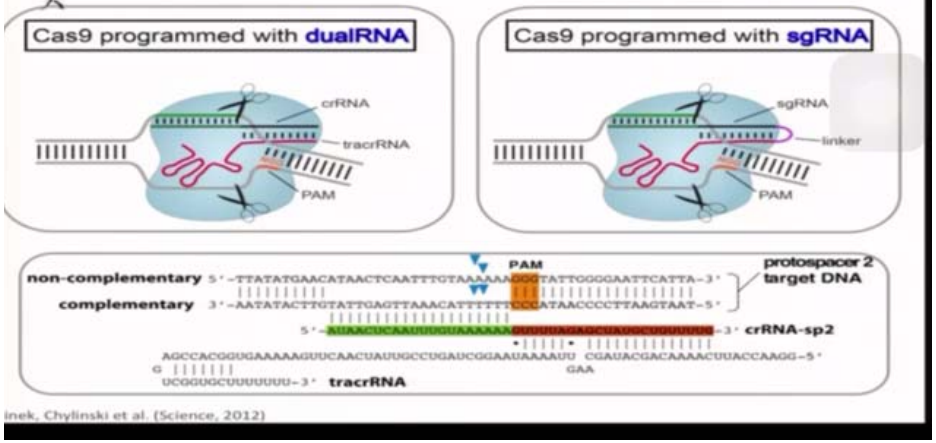
Type II CRISPR-Cas: model of crRNA maturation

- 2nd processing event: unknown exo- or endonuclease



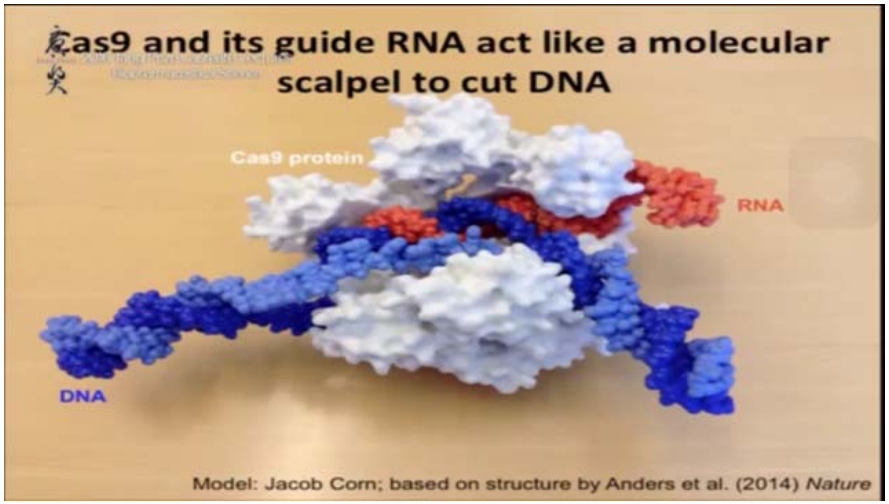
Deltcheva et al. (Nature, 2011)

CRISPR-Cas9: RNA-programmable DNA cleavage

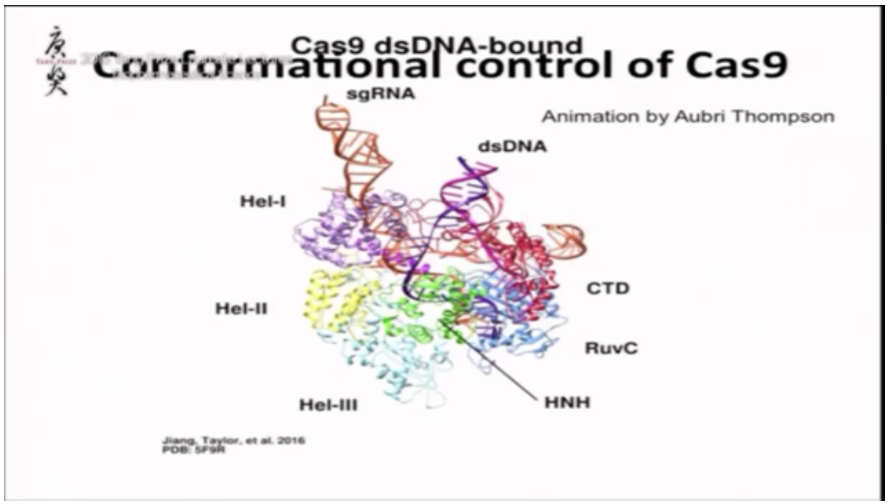


inek, Chylinski et al. (Science, 2012)

Cas9 and its guide RNA act like a molecular scalpel to cut DNA

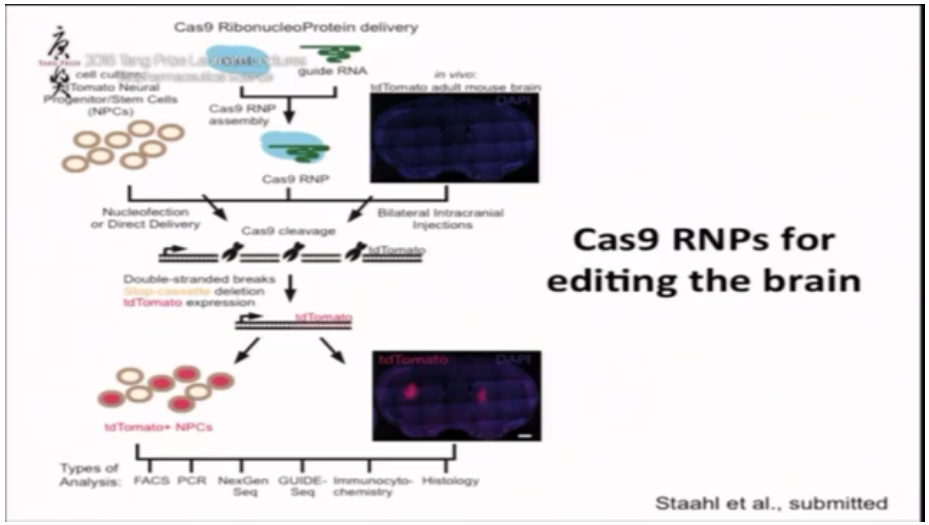


Conformational control of Cas9

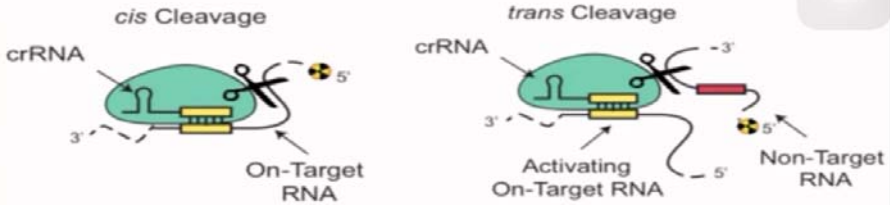


Current challenges to therapeutic applications of genome engineering

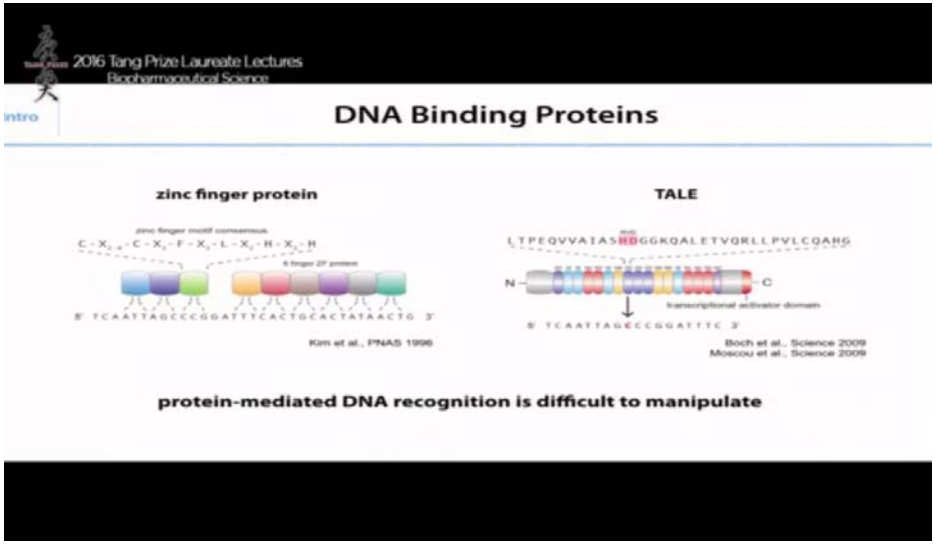
- Delivery
- Controlling repair pathways
- Ethics

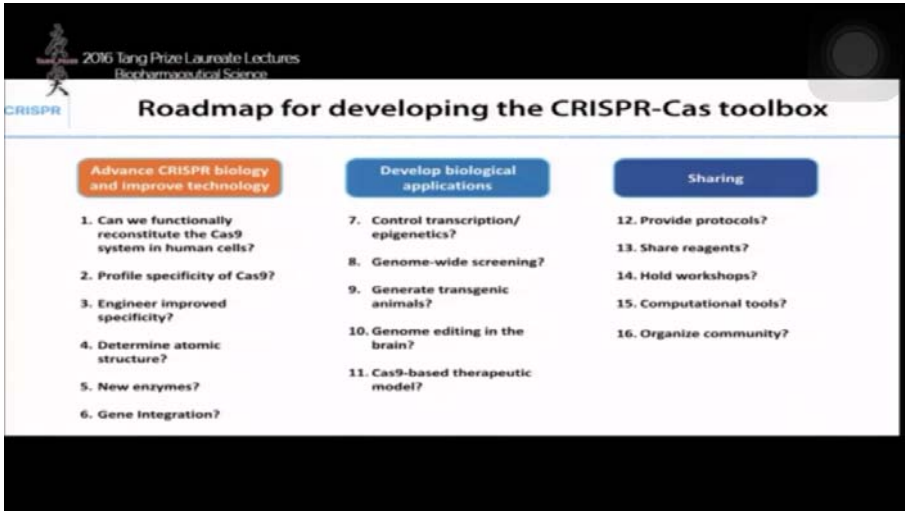


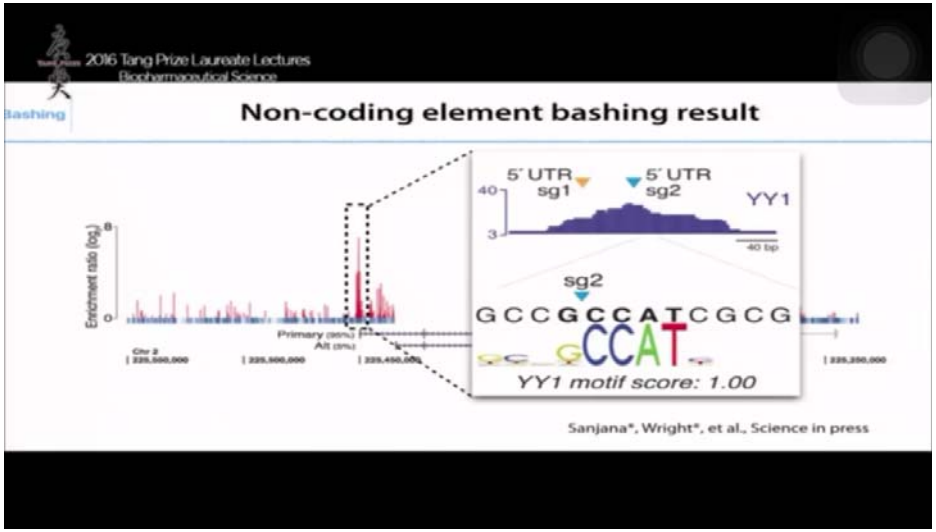
C2c2 is an RNA-activated RNase

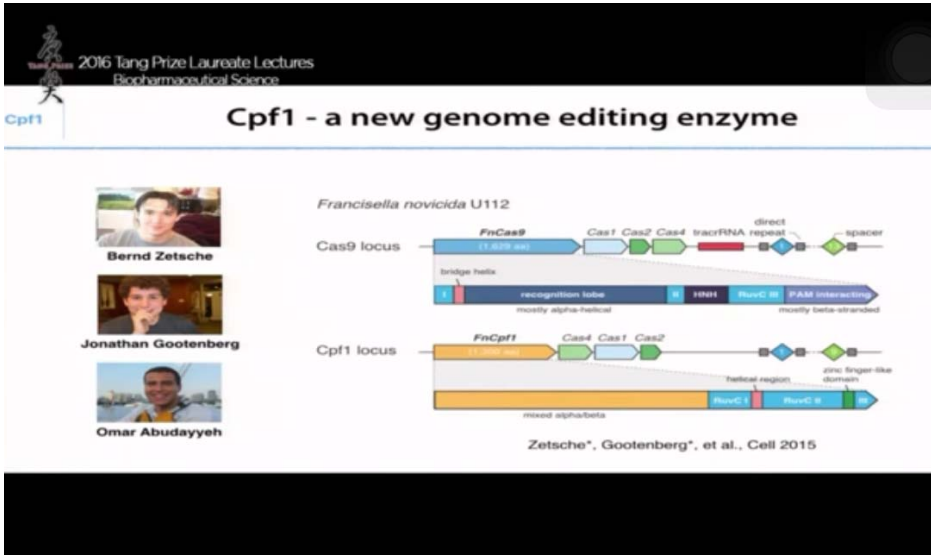


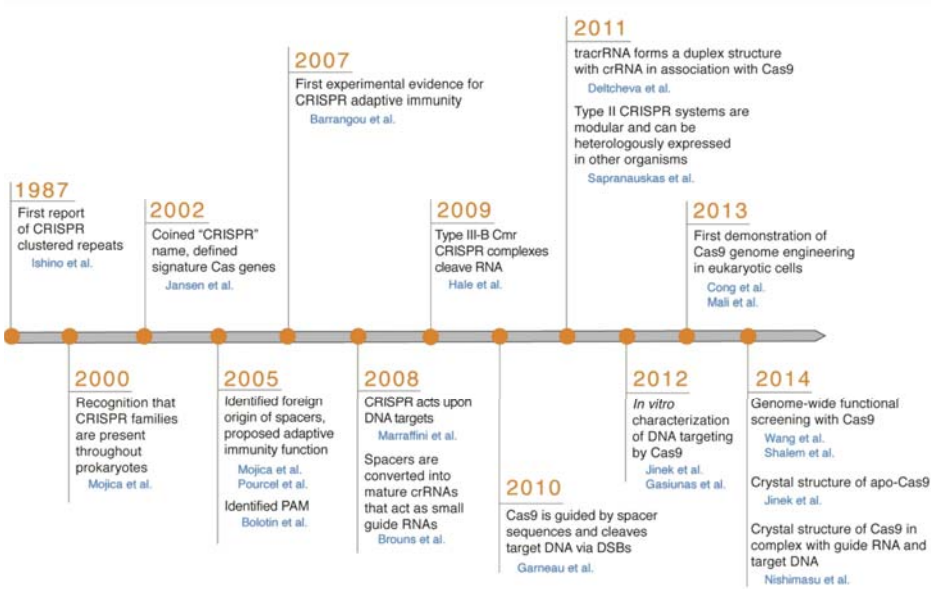

2016 Tang Prize Laureate Lectures
Biopharmaceutical Science
TANG PRIZE


2016 Tang Prize Laureate Lectures
Biopharmaceutical Science
Intro
DNA Binding Proteins
zinc finger protein
TALE
Kim et al., PNAS 1996
Boch et al., Science 2009
Moscou et al., Science 2009
protein-mediated DNA recognition is difficult to manipulate


2016 Tang Prize Laureate Lectures
Biopharmaceutical Science
CRISPR
Roadmap for developing the CRISPR-Cas toolbox
Advance CRISPR biology and improve technology
1. Can we functionally reconstitute the Cas9 system in human cells?
2. Profile specificity of Cas9?
3. Engineer improved specificity?
4. Determine atomic structure?
5. New enzymes?
6. Gene Integration?
Develop biological applications
7. Control transcription/ epigenetics?
8. Genome-wide screening?
9. Generate transgenic animals?
10. Genome editing in the brain?
11. Cas9-based therapeutic model?
Sharing
12. Provide protocols?
13. Share reagents?
14. Hold workshops?
15. Computational tools?
16. Organize community?

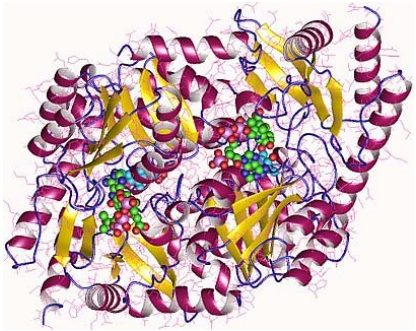

2016 Tang Prize Laureate Lectures
Biopharmaceutical Science
Bashing
Non-coding element bashing result
5′ UTR sg1
5′ UTR sg2
YY1
sg2
YY1 motif score: 1.00
Sanjana*, Wright*, et al., Science in press



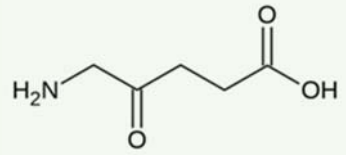


Enzyme (EC 2.3.1.37) Aminolevulinic acid synthase (ALAS)

- A key enzyme involves in C4 pathway
- ALAS is corresponding to cell growth dut to the regulation of hemB which is the essential gene.
- A enzyme needs co-factor, pyridoxal 5'-phosphate (PLP) whose role in this synthesis is detailed in the image



What is 5-Aminolevulinic Acid (5-ALA)?



5-Aminolevulinic acid (5-ALA)

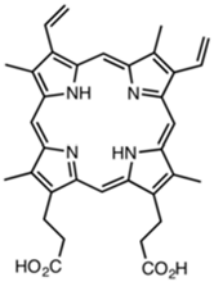
- Non-proteinogenic amino acid
- Essential intermediate in organisms

Promoting factor for crops

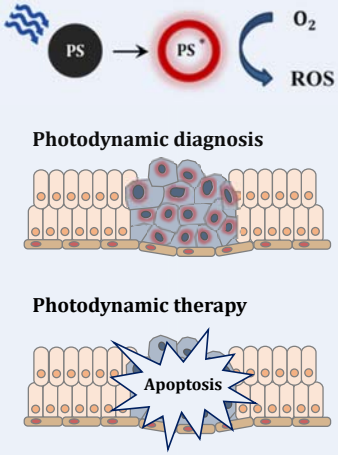
- **Production**
- **Tolerance**
  - **Salinity**
  - **Coldness**

Liu et al., *Appl. Microbiol. Biotechnol,* 2014, *98.17*, 7349-7357

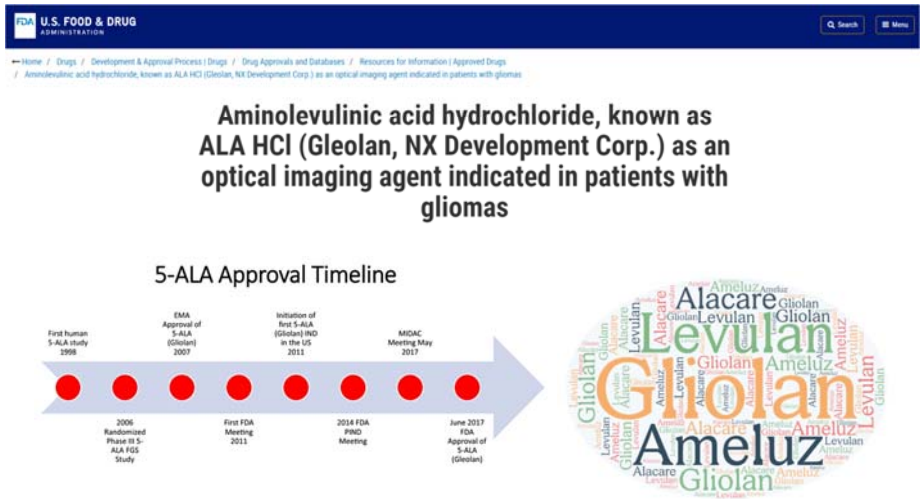
Promising prodrug for photodynamic therapy



Protoporphyrin IX (PPIX)
Photosensitizer (PS)



5-ALA has been approved for clinical treatment



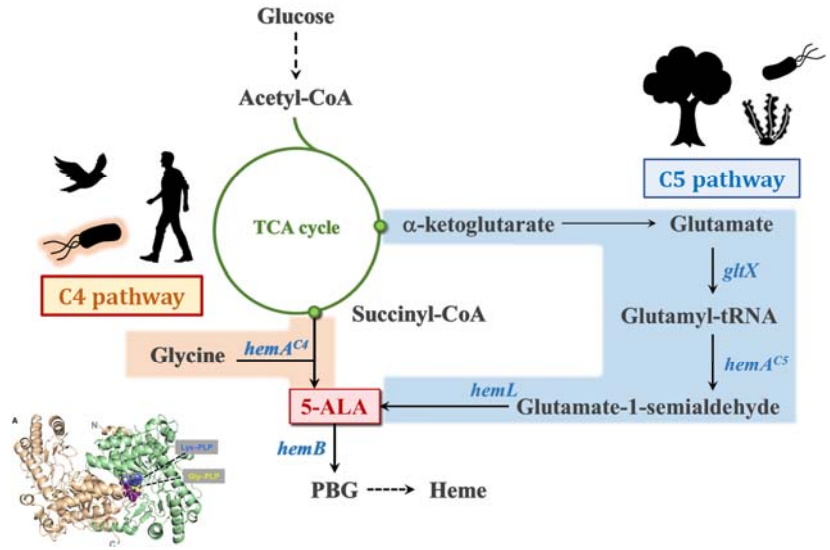
Hadjipanayis & Stummer. *J. Neuro-Oncology*, 2019, *141.3*, 479-486.

5-ALA has been approved for clinical treatment

Chemical			
Product	Description	Manufacturer	Price
5-ALA·HCl	For biochemistry	Millipore	NTD 3200/500 mg
5-ALA·HCl	≥97.0% (AT)	Sigma-Aldrich	NTD 7300/1 g
5-ALA·HCl	≥98%	Sigma-Aldrich	NTD 6400/500 mg
5-ALA·HCl	BioReagent, suitable for cell culture, powder, ≥98%	Sigma-Aldrich	NTD 3200/500 mg
Pharmaceutical			
Alacare	8 mg medicated plaster	Gebro Pharma AG	—
Ameluz	78 mg/g gel	Biofrontera	—
Gliolan	30 mg/mL powder for oral solution	IDT Biologika	NTD 80000 according to charge standard of Chang Gung Medical Foundation
Levulan kerastick	20% for topical solution	DUSA Pharmaceuticals, Inc.	USD 412

High value-added compound!

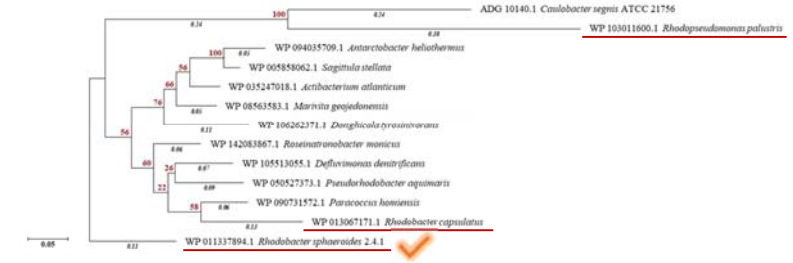
Biosynthesis Pathway of 5-ALA



ALA synthase (ALAS)

Selected gene at the beginning

Genes of interest | *RshemA*



Appl Microbiol Biotechnol (2007) 75:773–782
DOI 10.1007/s00253-007-0887-y

BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

5-Aminolevulinate production with recombinant *Escherichia coli* using a rare codon optimizer host strain

Weiqi Fu · Jianping Lin · Peilin Cen

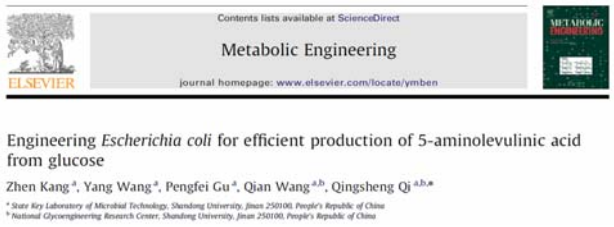
Metabolic Engineering of *Corynebacterium glutamicum* for Efficient Production of 5-Aminolevulinic Acid

BIOTECHNOLOGY AND BIOENGINEERING

Lili Feng,[1,2,3] Ya Zhang,[1,2,3] Jing Fu,[1,2,3] Yufeng Mao,[1,2,3] Tao Chen,[1,2,3] Xueming Zhao,[1,2,3] Zhiwen Wang[1,2,3]

RhtA: a non-specific ALA exporter

Genes of interest | *RshemA* | *rhtA*



Contents lists available at ScienceDirect

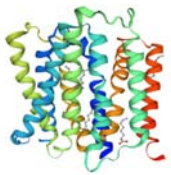
Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Engineering *Escherichia coli* for efficient production of 5-aminolevulinic acid from glucose

Zhen Kang[a], Yang Wang[a], Pengfei Gu[a], Qian Wang[a,b], Qingsheng Qi[a,b,*]

[a] State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China
[b] National Glycoengineering Research Center, Shandong University, Jinan 250100, People's Republic of China



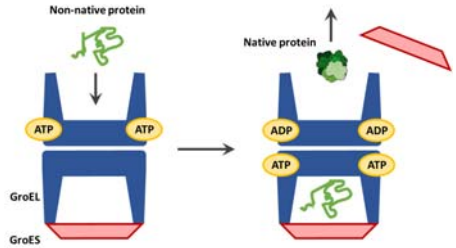
RhtA

Threonine/homoserine exporter

An integral membrane protein was reported that it was beneficial for efflux of ALA

One of the research

Genes of interest | *RshemA* | *rhtA* | *groES* | *groEL*

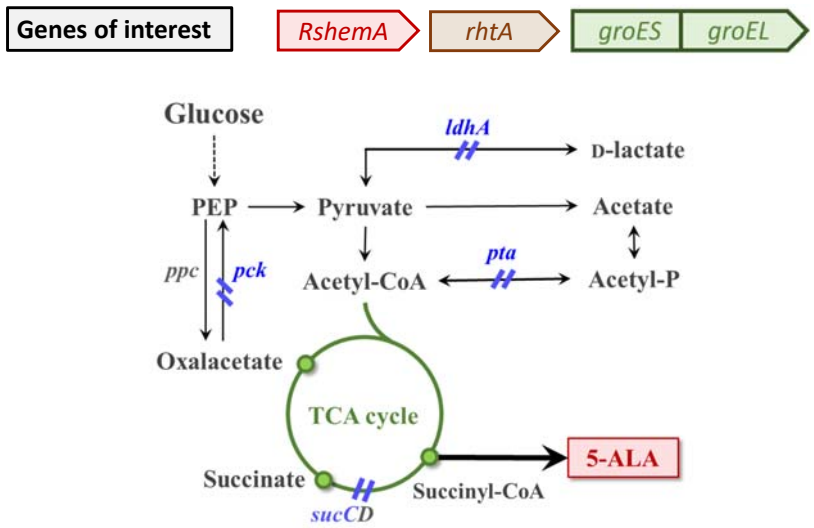


Heat shock protein (Hsp)

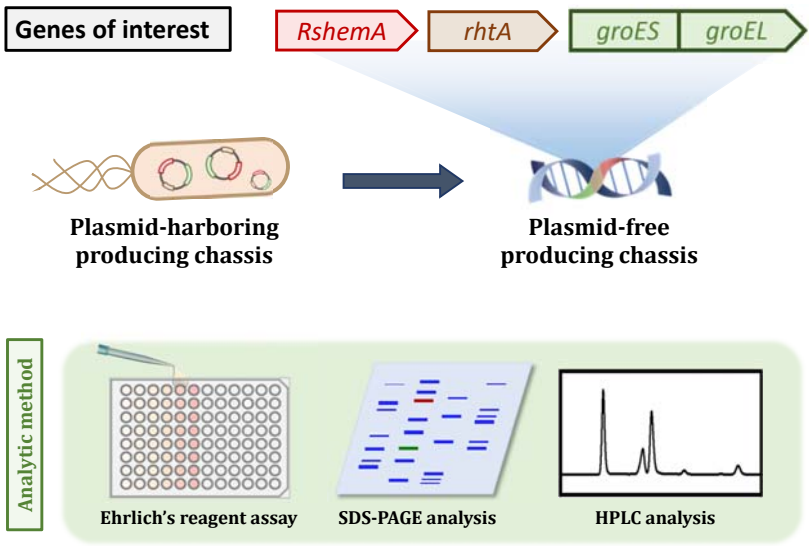
GroESL (Hsp60/Hsp10)

- First identified due to its importance for lambda phage biogenesis.
- Refold the proteins back to their native conformation.

Pathway of production ALA from glucsoe



Integrative process to obtain higher ALA




Journal of Bioscience and Bioengineering
VOL. 129 No. 4, 387–394, 2020
www.elsevier.com/locate/jbiosc
ELSEVIER

Quantification, regulation and production of 5-aminolevulinic acid by green fluorescent protein in recombinant *Escherichia coli*

Shih-I. Tan, Shao-Chun You, I-Tai Shih, and I-Son Ng*

Department of Chemical Engineering, National Cheng Kung University, Tainan 701, Taiwan

Applied Biochemistry and Biotechnology
https://doi.org/10.1007/s12010-019-03178-9

Enhanced 5-Aminolevulinic Acid Production by Co-expression of Codon-Optimized *hemA* Gene with Chaperone in Genetic Engineered *Escherichia coli*

Tzu-Hsuan Yu[1] · Ying-Chen Yi[1] · I-Tai Shih[1] · I-Son Ng[1]

Received: 1 October 2019 / Accepted: 11 November 2019/Published online: 16 December 2019

What are the concerns of "cascade enzyme reaction"?

- Retro-Metabolic pathway
- Enzyme expression system
- Optimization in synergism
- Enzyme stability
- Reactant solubility
- .....

OUTLINE

1. Activity and characterization
   - Temperature, pH, Substrate…
2. Preparation and quantification
3. Physical purification
   - Precipitation
   - Ultra-filtration
   - Dialysis
4. Chromatography
   - Ion exchange, size exclusion, affinity

Journal of the Taiwan Institute of Chemical Engineers 61 (2016) 97–105

Contents lists available at ScienceDirect

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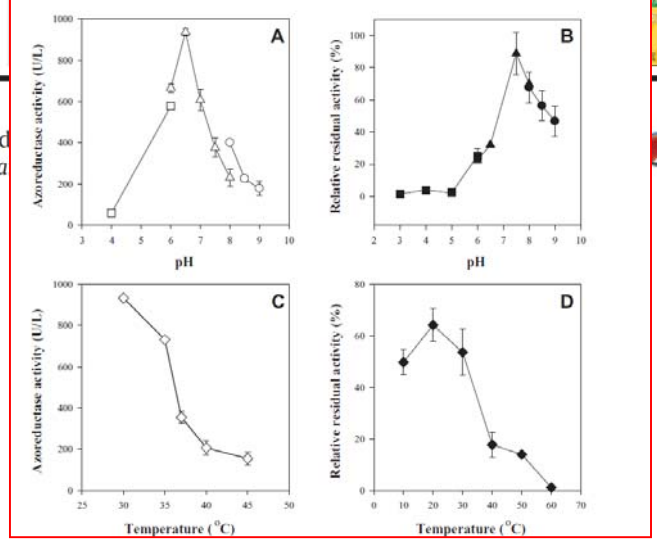
Cloning and
from *Shewa*



Some important concepts for sample preparation

1. A good sample preparation is the key to good result.
2. The protein composition of the cell lysate or tissue must be reflected in the patterns of 2-DE.
3. Avoid protein contamination from environment.
4. Co-analytical modification (CAM) must be avoided. (pre-purification sometimes leads to CAM)
5. Highly selective procedure for tissue analysis (Laser capture micro dissection, LCM)

Some important concepts for sample preparation

6. Treatment of sample must be kept to a minimum to avoid sample loss.
7. Keep sample as cold as possible.
8. Shorten processing time as short as possible.
9. Removal of salts
10. Minimized the unwanted processing, eg proteolytic degradation, chemical modification.

Frequently applied treatments

1. Cell washing
2. Cell disruption
3. Removal of contaminant
4. Microdialysis
5. Electrophretic desalting
6. Precipitation methods
7. For very hydrophobic protein

1. Cell washing

- To remove contaminant material.
- Frequent used buffer
  - PBS: phosphate buffer saline, sodium chloride, 145 mM (0.85%) in phosphate buffer, 150 mM pH7.2
  - Tris buffer sucrose (10mM Tris, 250 mM sucrose, pH 7.2)
- Enough osmoticum to avoid cell lysis

2. Cell disruption

- Gentle lysis method
  1. Osmotic lysis (cultured cells)
     - Suspend cells in hypoosmotic solution.
  2. Repeated freezing and thawing (bacteria)
     - Freeze using liquid nitrogen
  3. Detergent lysis (yeast and fungi)
     - Lysis buffer (containing urea and detergent)
     - SDS (have to be removed before IEF)
  4. Enzymatic lysis (plant, bacteria, fungi)
     - Lysomzyme (bacteria)
     - Cellulose and pectinase (plant)
     - Lyticase (yeast)

2. Cell disruption (continued)

- Vigorous lysis method
  1. Sonication probe (cell suspension)
     - Avoid overheat, cool on ice between burst.
  2. French pressure (microorganism with cell wall)
     - Cells are lysed by shear force.
  3. Mortar and pestle (solid tissue, microorganism)
     - Grind solid tissue to fine powder with liquid nitrogen.
  4. Sample grinding kit (for small amount of sample)
     - For precious sample.
  5. Glass bead (cell suspension, microorganism)
     - Using abrasive vortexed bead to break cell walls.

2. Cell disruption (continued)

- Key variable for successful extraction from crude material

1. The method of cell lysis
2. The control of pH
3. The control of temperature
4. Avoidance of proteolytic degradation

3. Removal of contaminants

- Major type of contaminants:

1. DNA/RNA
2. Lipids
3. polysaccharides
4. Solid material
5. Salt

DNA/RNA contaminant

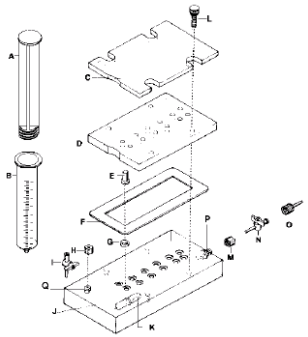
- DNA/RNA can be stained by silver staining.
- They cause horizontal streaking at the acidic part of the gel.
- They precipitate with the proteins when sample applying at basic end of IEF gel
- How to remove:
  1. precipitation of proteins
  2. DNase/RNase treatment
  3. sonication (mechanical breakage)
  4. DNA/RNA extraction method (phenol/chroloform)

Removal of other contaminants

- Removal of lipids:
  - >2% detergent
  - Precipitation
- Removal of polysaccharides:
  - Enzymatic procedure
  - Precipitation
- Removal of solid material
  - Centrifugation
- Removal of salts
  - Microdialysis
  - Precipitation

4. Microdialysis

- Specially design for small volume samples
- Membrane cut-off is about 8000 Da
- Drawbacks:
  - 1. Time consuming (some protease might be active and digest proteins during the dialysis)
  - 2. Some proteins precipitation after dialsis.



5. Electrophoretic desalting

- There are some case where the sample must not be dialysed. (halobacteria lysate)
- Some proteins will gel if desalted. (Bovine vitreous proteins)

Solution for above: low voltage (100V) for 5 hours before IEF running. (A. Gorg, 1995)

6. Precipitation methods.

- The reasons for applying protein precipitation procedure:

1. Concentrate low concentrated protein samples.
2. Removal of several disturbing material at the same time.
3. Inhibition of protease activity.

6. Five precipitation methods.

1. Ammonium sulfate precipitation
2. TCA precipitation
3. Acetone precipitation
4. TCA/Acetone precipitation
5. Ammonium acetate/method following phenol extraction

Ammonium sulfate precipitation

- Proteins tend to aggregate in high concentration of salt (salting out)
  - Add Ammonium sulfate slowly into solution and stir for 10-30 mins
  - Harvest protein by centrifugation.
- Limitation
  - Some proteins are soluble at high salt conc.
  - Ammonium sulfate seriously affect IEF.

TCA precipitation

- Trichloroacetic acid is a very affective protein precipitant.
  - Add TCA to extract to final conc.10-20%.
  - Add 10-20% TCA directly to tissue or cells.
  - Harvest protein by centrifugation.
  - Wash access TCA by ethanol or acetone.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

Acetone precipitation

- The most common organic solvent used to precipitated proteins, lipid and detergent remain in solution.
  - Add at least 3 vol. of ice-cold acetone into extract.
  - Stand on ice for at least 2 hours.
  - Harvest protein by centrifugation.
  - Remove access acetone by air drying.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - Some proteins would not precipitate.
  - DNA/RNA and glycan also precipitate.

TCA/acetone precipitation

- The method is more active than TCA or acetone alone. Most commonly used in 2-DE.
  - Suspension samples in 10% TCA/Acetone with 0.07% 2-mercaptoethanol or 20mM DTT.
  - Stand on -20C for at least 45mins.
  - Harvest protein by centrifugation.
  - Wash the pellet by acetone with0.07% 2-mercaptoethanol or 20mM DTT.
  - Remove access acetone by air dry.
- Limitation
  - Sometimes the pellet is hard to redissolve.
  - TCA must remove complete. (affecting IEF)
  - Some degradation or modification of protein occurs

Precipitation with ammonium acetate in methanol following phenol extraction

- The method is more suitable for plant sample with high level of interfering substance
  - Proteins are extracted into buffer saturated phenol.
  - Precipitated by ammonium acetate/methanol.
  - Harvest protein by centrifugation.
  - Wash with ammonium acetate/methanol followed by acetone.
- Limitation
  - Complicated.
  - Time consuming.

7. For very hydrophobic proteins

Membrane proteins do not easily go into solution. A lot of optimization work is required.

1. Thiourea procedure
2. SDS procedure
3. New zwitterionic detergent and sulfobetains

Thiourea procedure

7M urea + 2M thiourea (Rabilloud, 1998)

Pros: Increase spot number considerably.

Cons: Causing artifact spots.

Causing vertical streaking at acidic area.

SDS procedure

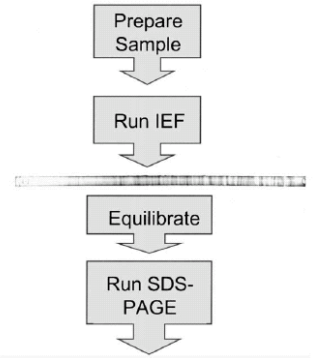
- For emergency case.
- Up to 2% SDS can be used.
- Have to dilute SDS samples at least 20 fold with urea an a non or zwitterionic detergent containing solutions.
- The major reasons for using SDS:
  1. Formation of oligomers can be prevented
  2. Dissolved tough cell walls samples (with boiling)
  3. Dissolved very hydrophobic proteins

New zwitter-ionic detergent and sulfo-betains

Three major types of detergent

1. Non ionic detergent
   - Triton x-100, Tween 20, Brij-35
2. Ionic detergent
   - SDS, CTAB, Digitonin
3. Zwittergent
   - CHAPS, CHAPSO, Zwittergent 3-08, 3-10, 3-12…

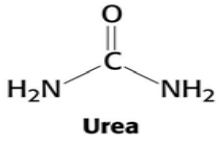
Now, we are ready to dissolve protein samples in IEF lysis buffer



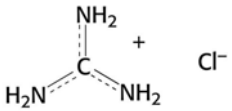
2-DE are in denaturing condition

Three components must present in 2-DE denaturing condition (namely, in IEF lysis buffer)

1. Urea (often > 7M)
2. Reductant (DTT used most widely)
3. Non-ionic or zwitterionic detergent
4. Dye



Urea



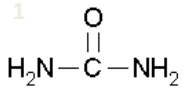
Guanidinium chloride

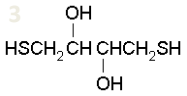
Why not using native condition?

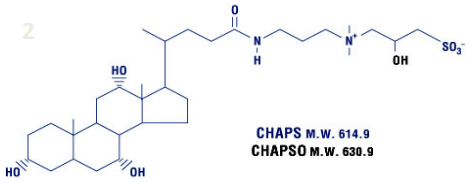
1. Under native condition, a great part of proteins exists in several conformations. This leads to more complex 2-DE patterns.
2. Native protein complexes sometimes too big to enter the gel.
3. Reduction of protein-protein interactions.
4. For match the theoretical pI and MW, all proteins should not have 3D structure or quanternary structure.

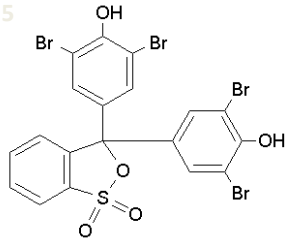
Composition of standard lysis buffer (for IEF)

1. 9M urea
2. 4% CHAPS
3. 1% DTT
4. 0.8% carrier ampholyte
5. 0.02% bromophenol blue.



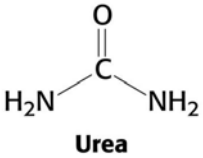


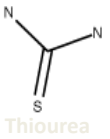




Functions of denaturant (Urea)

1. To convert proteins into single conformation by canceling 2nd and 3rd structure.
2. To keep hydrophobic proteins into solution.
3. To avoid protein-protein interaction.
4. Thio urea: for very hydrophobic proteins only.

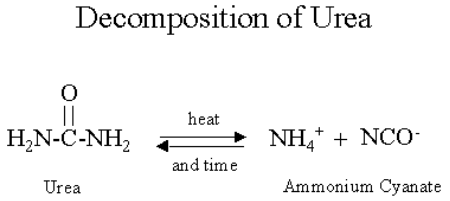


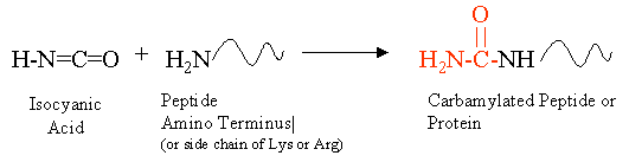


Beware when using urea

1. The purity of urea is very critical
2. Isocyanate impurities and heating will cause carbamylation of the proteins.
3. It does not seem to make a difference what grade of urea is used because, urea + heat + protein = carbamylation.

Carbamylation of proteins



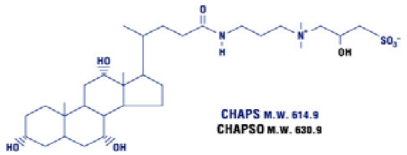


Results of Carbamylation

Amino Acid	Residue Composition	Residue Monoisotopic Mass	Delta Mass
Lysine	$C_6H_{12}N_2O$	128.09496	0
Carbamyl Lysine	$C_7H_{13}N_3O_2$	171.10078	43.00582
Carbamylation	* NHCO	43.00582	-
*Note: A proton is lost from the amino group on the protein during carbamylation and thus the change in composition is NHCO.			

Functions of detergent (CHAPS)

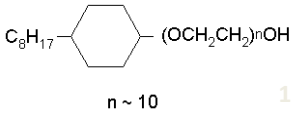
- To Combine all the advantages of polar, sulfobetaine-containing detergents and hydrophobic, bile salt, anionic detergents into a single molecule with superior membrane protein solubilization properties
- Non-denaturing
- Able to disrupt nonspecific protein interactions
- Less protein aggregation than non-ionic detergents
- Electrically neutral
- Easily removed by dialysis



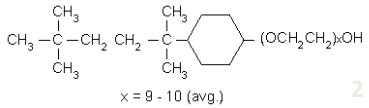
Other detergents

1. Triton X-100

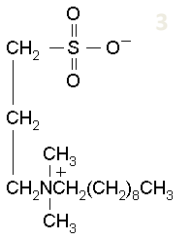
(not easily remove and interfering MS)



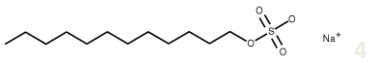
2. Nonidet NP-40



3. SB3-10

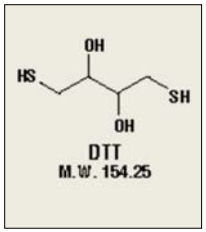


4. SDS



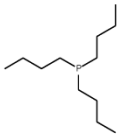
Functions of reductant

- To prevent different oxidation steps of proteins.
- 2-mercaptoethanol should not be used because its buffering effect above pH 8.
- Keratin contamination might from 2-mercaptoethanol.
- DTT (dithiothreitol) or DTE (dithioerythritol) are used widely.
- DTT and DTE ionized above pH8. They move toward anode during IEF in basic pH gradient.
- It leads to horizonal streaking at basic area.



Other reduction methods

- TBP (tributylphosphine): very unstable.



- An alternative way to adequate and reproducible 2-DE patterns in basic area:

1. Addition of higher amount of DTT to the gel
2. Addition of more DTT to a cathodal paper strip.

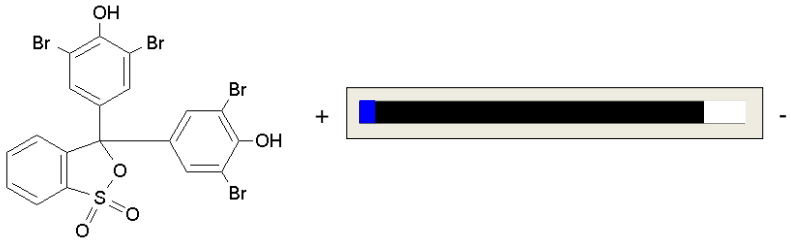
Function of carrier ampholyte

They do not disturb IEF like buffer addition, because they become uncharged when migrating to their pI.

1. To generate pH gradients
2. To substituting ionic buffer
3. To improve the solubility of protein
4. Dedicated pH intervals, prepared for the addition to immobilized pH gradients, are called IPG buffer.

Function of dyes

- To visualize the sample solution
- To monitor the 2-DE running condition.
- Bromophenol blue is interchangeable with Orange G.



Other considerations

- Protease inhibitors
  - 1. Some proteases are also active in presence of urea and detergent.
  - 2. PMSF is frequently used (8mM), toxic and short half-life.
  - 3. Pefabloc (AEBSF) can also be used but modified proteins.
  - 4. NO complete insurance against protease activity
  - 5. Boiling sample in SDS buffer for a few seconds can inactive protease.
  - 6. Precipitate proteins with TCA/acetone at -20C might inactivation protease activity.

Protease inhibitor	Effective against:	Limitations
PMSF (Phenylmethylsulfonyl fluoride) Most commonly used inhibitor. *Use at concentrations up to 1 mM.*	PMSF is an irreversible inhibitor that inactivates: • serine proteases • some cysteine proteases	PMSF rapidly becomes inactive in aqueous solutions: Prepare just prior to use. PMSF may be less effective in the presence of thiol reagents such as DTT or 2-mercapto-ethanol. This limitation can be overcome by disrupting the sample into PMSF-containing solution lacking thiol reagents. Thiol reagents can be added at a later stage. PMSF is very toxic.
AEBSF (Aminoethyl benzylsulfonyl fluoride or Pefabloc SC Serine Protease Inhibitor) *Use at concentrations up to 4 mM.*	AEBSF is similar to PMSF in its inhibitory activity, but is more soluble and less toxic.	AEBSF-induced modifications can potentially alter the pI of a protein.
1 mM EDTA or 1 mM EGTA *Generally used at 1 mM.*	These compounds inhibit metalloproteases by chelating free metal ions required for activity.	
Peptide protease inhibitors (e.g. leupeptin, pepstatin, aprotinin, bestatin) • reversible inhibitors • active in the presence of DTT • active at low concentrations under a variety of conditions *Use at 2–20 µg/ml.*	Leupeptin inhibits many serine and cysteine proteases. Pepstatin inhibits aspartyl proteases (e.g. acidic proteases such as pepsin) Aprotinin inhibits many serine proteases. Bestatin inhibits aminopeptidases.	Peptide protease inhibitors are: • expensive. • small peptides and thus may appear on the 2-D map, depending on the size range separated by the second-dimension gel. Pepstatin does not inhibit any proteases that are active at pH 9.
TLCK, TPCK (Tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone) *Use at 0.1–0.5 mM.*	These similar compounds irreversibly inhibit many serine and cysteine proteases.	
Benzamidine *Use at 1–3 mM.*	Benzamidine inhibits serine proteases.	

Other considerations

- Alkaline condition

Tris base (40mM) or spermidine (25mM) sometimes add to lysis buffer to maximize protein extraction.

– Pros:

1. They can also precipitate DNA/RNA.
2. They keep protease activity low.

– Cons:

1. Precipitation of basic protein.
2. Ionic contamination is to high.

Before running IEF, you should…

- Measure the protein conc. in your samples.

– Widely used protein assay methods

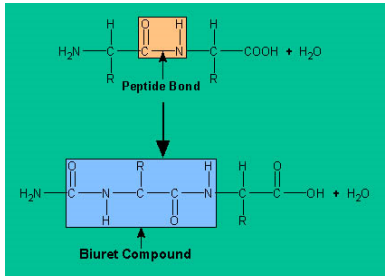
1. Biuret
2. Lowry methods.
3. Bradford methods.
4. UV methods.
5. Special methods
6. Other commercial methods.
    1. BCA assay (bicinchoninic acid assay, Pierce)
    2. DC protein assay (detergent compatible, Bio-rad)
    3. DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

1. Biuret method

– Principle: The reactivity of the peptide bonds with the copper [II] ions under alkaline conditions to form purple biuret complex.

– Interfering substance: Ammonium sulfate, Tris, etc.

– Sensitivity: >mg



A white, crystalline, nitrogenous substance, C2O2N3H5, formed by heating urea. It is intermediate between urea and cyanuric acid.

2. Lowry method

- Principle: The reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Try, Try).
- Interfering substance: amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds.
- Sensitivity: > 0.1 mg

3. Bradford method

- Principle: The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie® dye binds primarily with basic and aromatic side chains. The interaction with arginine is very strong and less strong with histidine, lysine, tyrosine, tryptophan, and phenylalanine. About 1.5 to 3 molecules of dye bind per positive charge on the protein.
- Interfering substance: amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids, ammonium ions, zwitterionic buffers, nonionic buffers and thiol compounds.
- Sensitivity: >10 -100 ug

4. UV methods

- Principle: The aromatic groups (Phe, Tyr, Trp) and the peptide bonds have maximum UV absorbance around 280nm and 200nm. 280nm was used most frequently.
- Interfering substance: anything containing
- Sensitivity: >mg

5. Special methods

- Principle: Some proteins contain functional groups, eg: Heme in peroidase, hemoglobin and transferrin can be detected at 403nm, Cd2+ in some phytochelatins.
- Interfering substance: similar functional groups.
- Sensitivity: various

6. Commercial methods

A. BCA assay (bicinchoninic acid assay, Pierce)

This process is a two-step reaction.

Protein + Cu^{2+} + OH^- ⟶ Cu^{1+}

Cu^{1+} + 2 BCA ⟶ Cu^{1+}/BCA chromophore (562 nm).

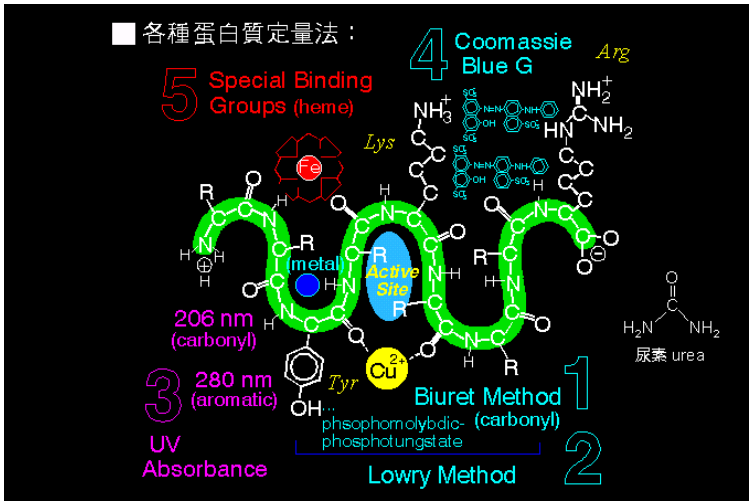
B. DC protein assay (detergent compatible, Bio-rad)

C. DC/RC protein assay (detergent/reducing agent compatible, Bio-rad)

Physical properties of protein or enzyme

- Molecular weight
- pI (isoelectric point)
- Hydrophobicity
- Disulfide bond
- ....

Quantification



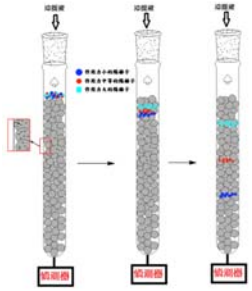
Adapted from Juang in NTU

Purification: chromatography

- Ion exchange
  - Anion
  - Cation
  - pH effect
  - Salt effect
- Affinity (His-trap and other tag)
  - Genetic engineering
  - Buffer conc.
  - Ultrafiltration
- Size exclusion (desalting)
  - Ultrafiltration
- Hydrophobicity interaction (HIC)

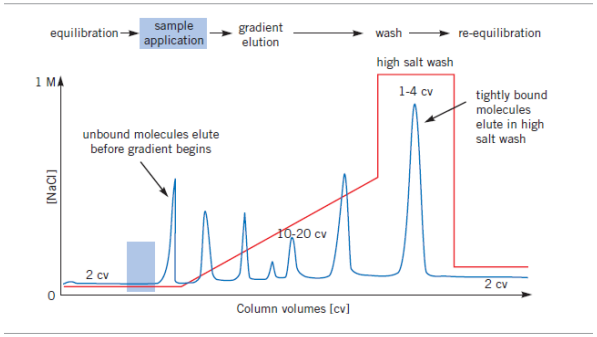
Ion exchange introduction

- Resin property
- Column selection
- Mobile phase
  - pH and salt effect



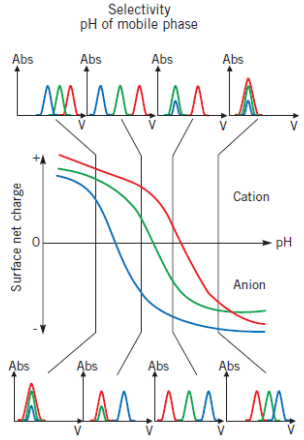
藉由待測離子與固定相上帶相反電荷之官能基兩者之間的庫倫作用力(Coulombic interaction)來結合、再利用移動相使目標離子脫離而被沖提出來，再度形成自由離子。待測物質的滯留時間取決於目標離子和固定相（stationary phase，一般稱為管柱，因固定相會填充在空心管中）所填充的離子交換樹脂兩者之間的親和力大小，而影響的因素如：離子的電荷數、離子半徑、質量等等。因親和力越大的離子越難沖提，造成滯留時間不同、並可以形成不同的離子群達到分離的效果，當移動相通過偵測器時，便可進行定性及定量的分析。

Ion exchange purification



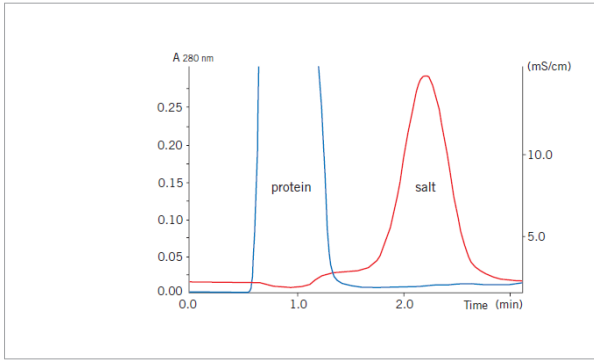
EX gradient elution.

Gradient elution



pH effect

For example: desalting



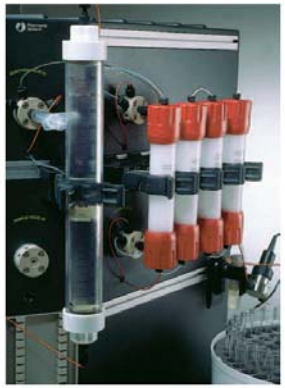


Fig 7. Buffer exchange of mouse plasma (10 ml) on HiPrep™ 26/10 Desalting.

Protein purified by HIC

System:	ÄKTAexplorer
Sample:	Fab fraction from STREAMLINE SP, 2 ml
Columns:	HiTrap HIC Selection Kit (1 ml columns):
	HiTrap Phenyl HP
	HiTrap Phenyl FF (low sub)
	HiTrap Phenyl FF (high sub)
	HiTrap Butyl FF
	HiTrap Octyl FF
Binding buffer:	1 ml $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Gradient:	20 column volumes from 0–100% elution buffer
Flow rate:	2 ml/min (300 cm/h)

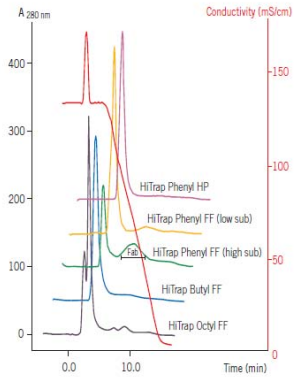
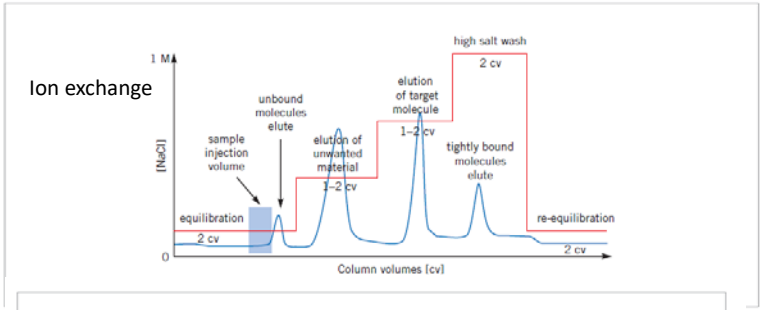


Fig. 32. HIC media scouting using HiTrap HIC Selection Kit.

Stepwise process

Ion exchange



HIC

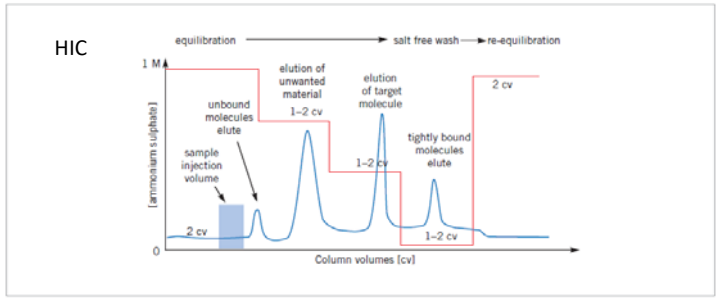


Fig. 45. Step elution.

Typical affinity purification

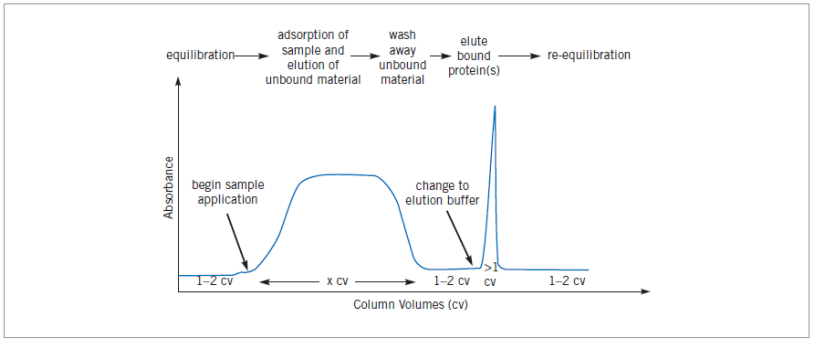
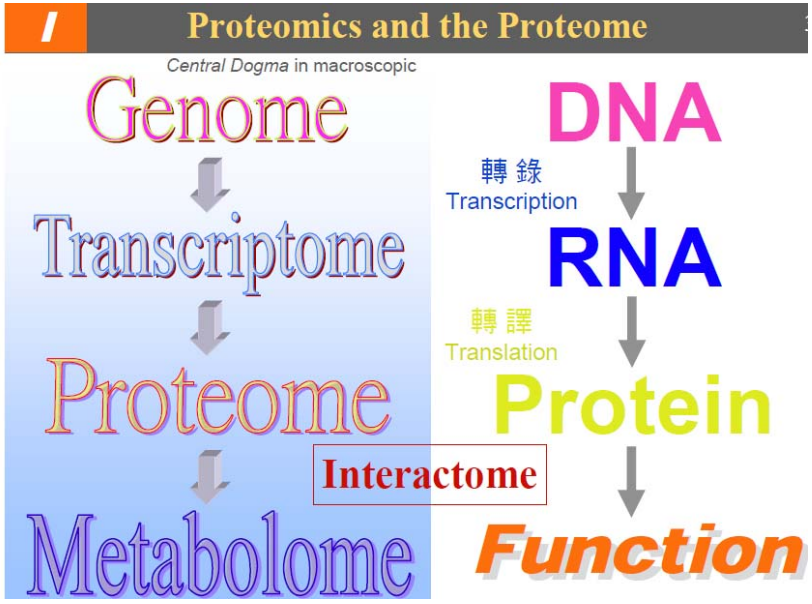


Fig. 40. Typical affinity purification.

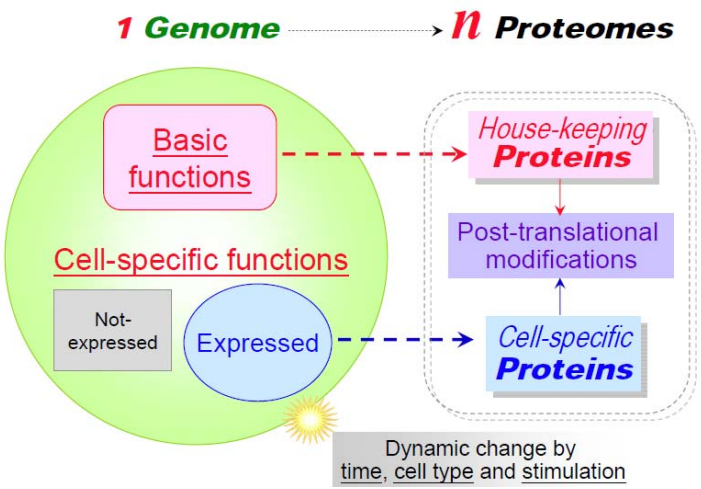
Enzymes and Fermentation Technology

Introduction of proteome and proteomics approach

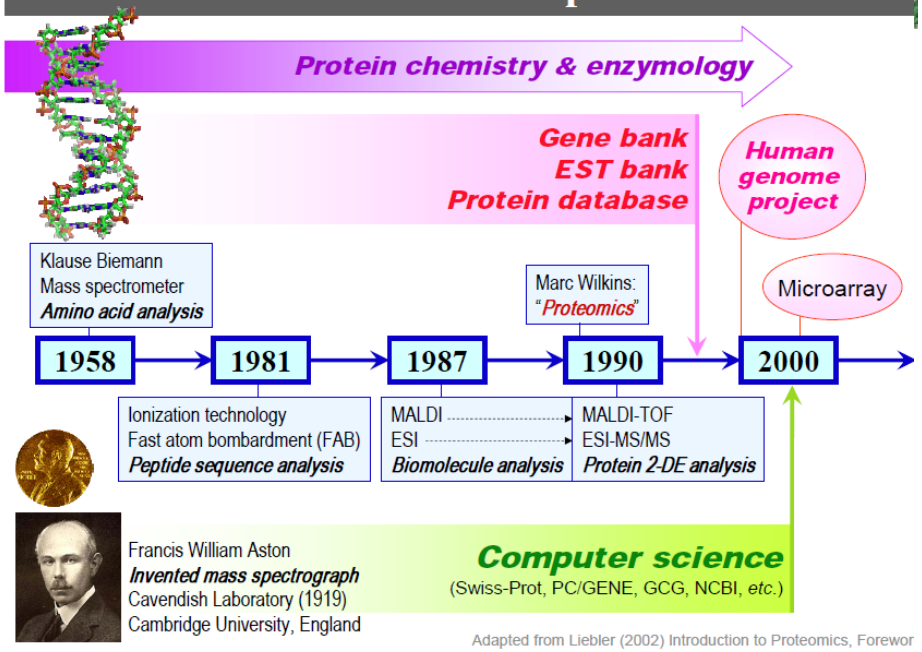
Central dogma



Between Genome and Proteomes



Historical review for proteomics



Adapted from Liebler (2002) Introduction to Proteomics, Forewor

Comparison between traditional protein analysis and proteomics

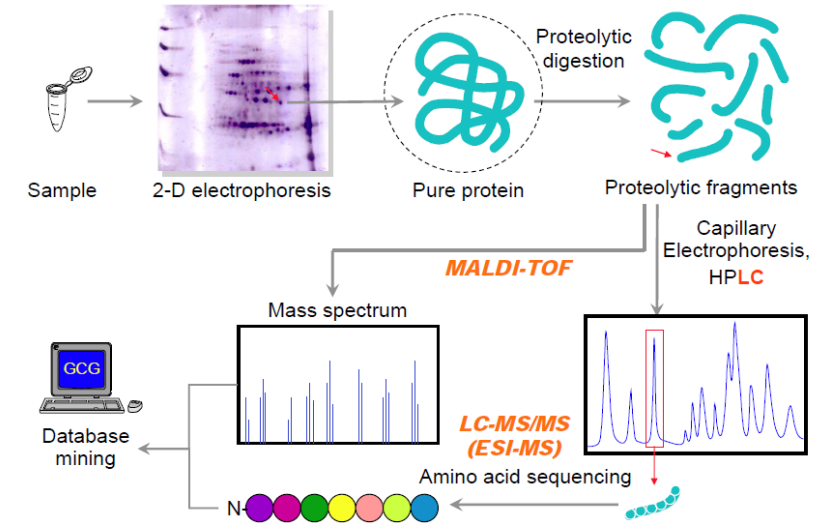
Is proteomics just what we used to call *protein chemistry*

Protein chemistry	*Proteomics*
Individual proteins	Complex mixtures
Complete sequence analysis	Partial sequence analysis
Emphasis on structure and function	On identification from database
Structural biology	Systems biology
Fixed protein target	Dynamic proteomic targets
Traditional technology	High-through put technology

陸軍步兵之巷戰佔領　　空軍之地毯式轟炸

Genomics and proteomics have challenged biologists to think Big

Quick identification of an unknown protein



Adapted from Juang in NTU

Two dimensional electrophoresis

- Only "Proteomics" is the large-scale screening of the proteins of a cell, organism or biological fluid, a process which requires stringently controlled steps of sample preparation, 2-D electrophoresis, image detection and analysis, spot identification, and database searches.
- The core technology of proteomics is 2-DE
- At present, there is no other technique that is capable of simultaneously resolving thousands of proteins in one separation procedure. (sited in 2000)

Evolution of 2-DE methodology

Traditional IEF procedure:

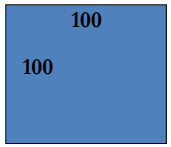
- IEF in run in thin polyacrylamide gel rods in glass or plastic tubes.
- Gel rods containing: 1. urea, 2. detergent, 3. reductant, and 4. carrier ampholytes (form pH gradient).
- Problem: 1. tedious. 2. not reproducible.

In the past

Evolution of 2-DE methodology

SDS-PAGE Gel size:

- This "O'Farrell" techniques has been used for 20 years without major modification.
- 20 x 20 cm have become a standard for 2-DE.
- Assumption: 100 bands can be resolved by 20 cm long 1-DE.
- Therefore, 20 x 20 cm gel can resolved 100 x 100 = 10,000 proteins, in theory.



Evolution of 2-DE methodology

Problems with traditional 1st dimension IEF

- Works well for native protein, not good for denaturing proteins, because:

OPERATOR DEPENDENT

1. Takes longer time to run.
2. Techniques are cumbersome. (the soft, thin, long gel rods needs excellent experiment technique)
3. Batch to batch variation of carrier ampholytes.
4. Patterns are not reproducible enough.
5. Lost of most basic proteins and some acidic protein.

Evolution of 2-DE methodology

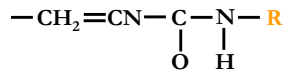
Resolution for IEF: Immobilized pH gradients.

- Developed by Bjellqvist (1982, Biochem. Biophys Methods, vol 6, p317)
- PH gradient are prepared by co-polymerizing acrylamide monomers with acrylamide derivatives containing carboxylic and tertiary amino groups.

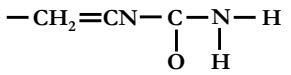
1. The pH gradient is fixed, not affected by sample composition.
2. Reproducible data are presented.
3. Modified by Angelika Gorg by using thin film to support the thin polyacrylamide IEF gel, named Strips. (1988, Electrophoresis, vol 9, p 531)

II. Immobilized pH gradient, IPG

- First developed by Righetti ,(1990).
- Immobilized pH gradient generated by buffering acrylamide derivatives (Immobilines)
- Immobilines are weak acid or weak base.
- General structure

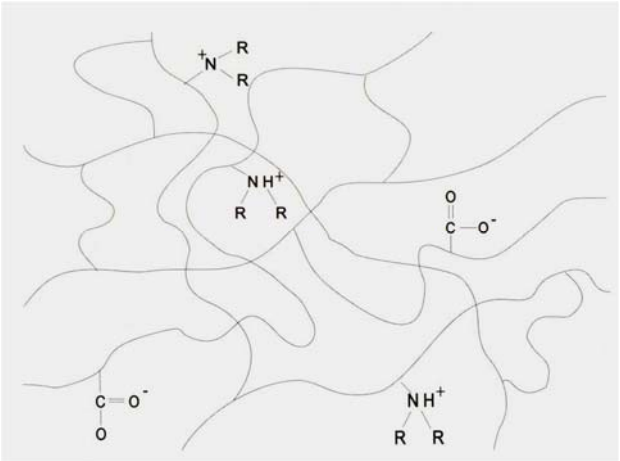


R = amino or carboxylic groups

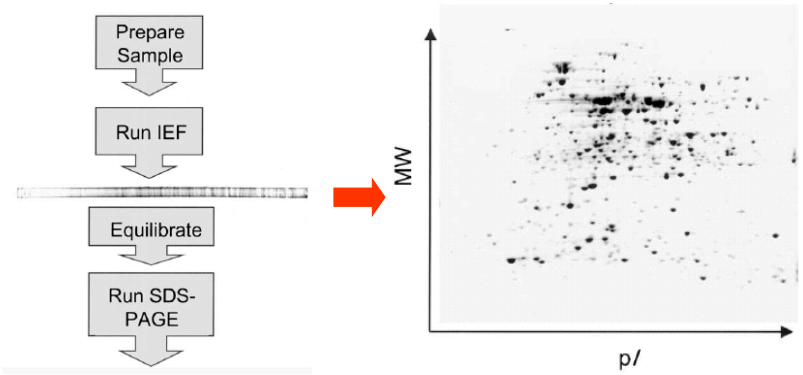


Acrylamide

Schematic drawing of a IPG



Run 2-DE, a quick overview

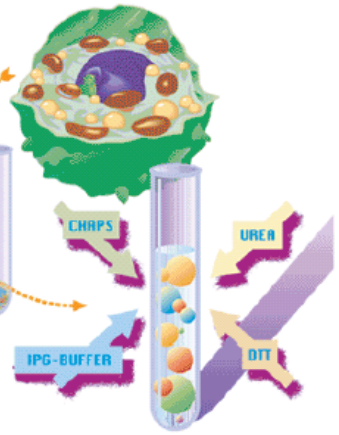


Run 2-DE, step by step

1. Prepare the sample

Samples are denatured and fully solubilized for optimal separation.

Solubilize your cell extract or sample in urea, a non-ionic detergent, IPG-Buffer and a reducing agent.



Run 2-DE step by step

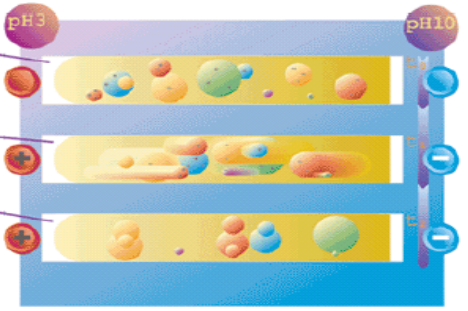
2. Run the first dimension – IEF

In the first dimension, proteins separate by isoelectric point (pI) in the immobilized pH gradient (IPG) of the Immobiline DryStrip gel.

A. Apply the sample to the IPG strip either in a defined zone or distributed throughout the entire strip.

B. Apply the electric field. The charged proteins migrate within the pH gradient.

C. When the proteins reach the pH in the gradient equal to their pIs, their net charge drops to zero and they focus as distinct zones. Because the pH gradient in the strip is immobilized, no further migration can occur.



Run 2-DE step by step

3. Run the second dimension – SDS-PAGE

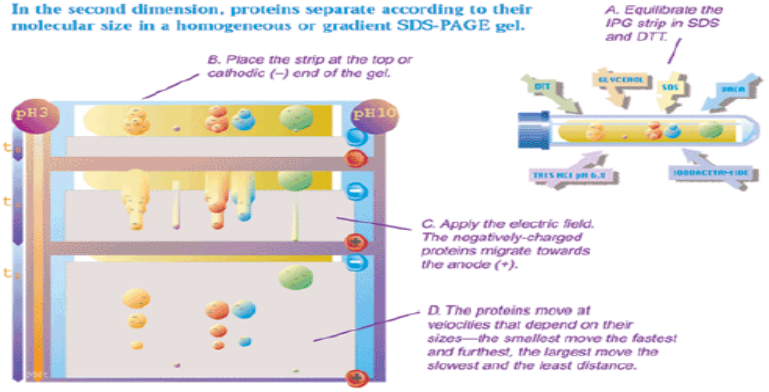
In the second dimension, proteins separate according to their molecular size in a homogeneous or gradient SDS-PAGE gel.

A. Equilibrate the IPG strip in SDS and DTT.

B. Place the strip at the top of cathodic (–) end of the gel.

C. Apply the electric field. The negatively-charged proteins migrate towards the anode (+).

D. The proteins move at velocities that depend on their sizes—the smallest move the fastest and furthest, the largest move the slowest and the least distance.



Run 2-DE step by step

4. Visualize and analyze

Detect separated proteins by autoradiography, staining, or immunodetection after blotting onto a membrane. An array of powerful tools and techniques is available to compare samples and identify proteins of interest:

- Image analysis software to determine spot position and abundance, compare gel images, create databases, and search for patterns;
- Mass spectrometry to determine masses with high precision, peptide fragment fingerprints, amino acid sequences, and nature and site of post-translational modifications.

Todays 2-DE

- Only high-resolution 2-DE with both dimensions run under denaturing conditions is used.
- Native 2-DE plays no big role.
- Goal: to separate and display all gene products present.

Challenges for 2-DE

1. Spot number:
   - 10,000-150,000 gene products in a cell.
   - PTM makes it difficult to predict real number.
   - Sensitivity and dynamic range of 2-DE must be adquete.
   - It's imposssible to display all proteins in one singel gels.

Challenges for 2-DE

2. Isoelectric point spectrum:
   - pI of proteins: range from pH 3-13. (by in vitro translated ORF)
   - PTM would not alter the pI outside this range.
   - pH gradient from 3-13 dose not exist.
   - For proteins which pI > 11.5, they need to be handed separately.

Challenges for 2-DE

3. molecular weights:
   - Small proteins or peptides can be analyzed by modifying the gel and buffer condition of SDS-PAGE.
   - Protein > 250 kDa do not enter 2nd SDS-PAGE properly.
   - 1-DE (SDS-PAGE) can be run in a lane at the side of 2-DE.

Challenges for 2-DE

4. hydrophobic proteins:

- Some very hydrophobic proteins do not go in solution.
- Some hydrophobic proteins are lost during sample preparation and IEF.
- More chemical developments are required.

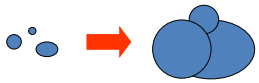
Challenges for 2-DE

5. Sensitivity of detection:

- Low copy number proteins are very difficult to detect, even employing most sensitive staining methods.
- Sensitivity of staining methods:
  1. Silver staining
  2. Fluorescent staining
  3. Dye binding staining (CBR)

Challenges for 2-DE

6. Loading capacity:



- For detection of low abundant proteins, more sample needs to be loaded.
- A wide dynamic range of the SDS-PAGE is required to prevent merging of highly abundant protein.
- Loading capacity: IEF > SDS-PAGE.

Challenges for 2-DE

7. Quantitation:

- The detection method must give reliable quantitative information.
- Silver staining does not give reliable quantitative data.

Challenges for 2-DE

8. Reproducibility:

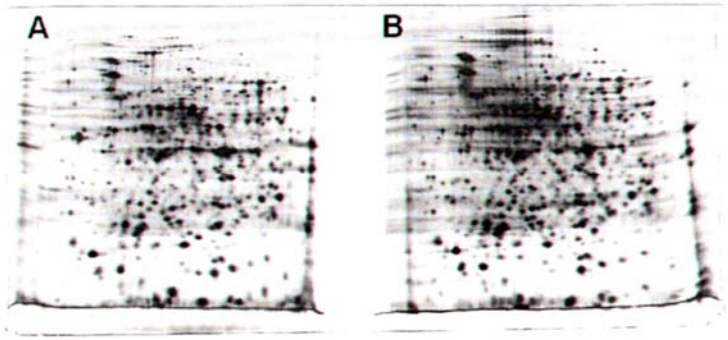
- Highest importance in 2-DE experiment.
- Immobilized pH gradient strip have improved a lot for 1st dimension consistency
- Variation most comes from sample preparation.

A good-looking spot pattern :
streak and smear free
is not a guarantee for best 2-DE protocol.

Some important concepts for sample preparation

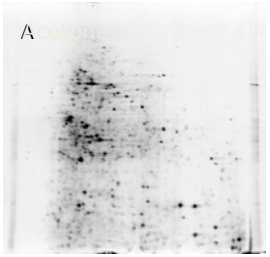
6. Treatment of sample must be kept to a minimum to avoid sample loss.
7. Keep sample as cold as possible.
8. Shorten processing time as short as possible.
9. Removal of salts
10. Minimized the unwanted processing, eg proteolytic degradation, chemical modification.

Example, Acetone precipitation

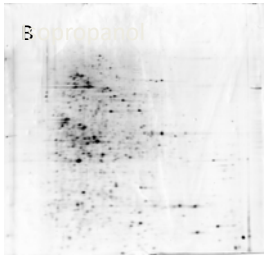


With Acetone precipitation

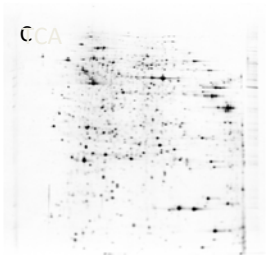
Crude extract by lysis buffer



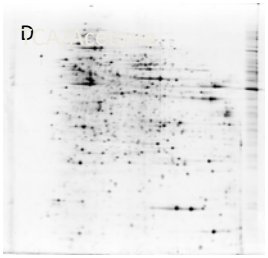
Σ 823 spots



Σ 757 spots

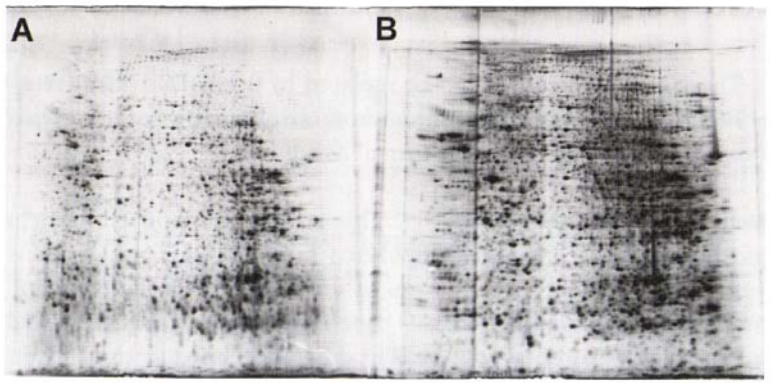


Σ 969 spots



Σ 899 spots

Example: thiourea procedure

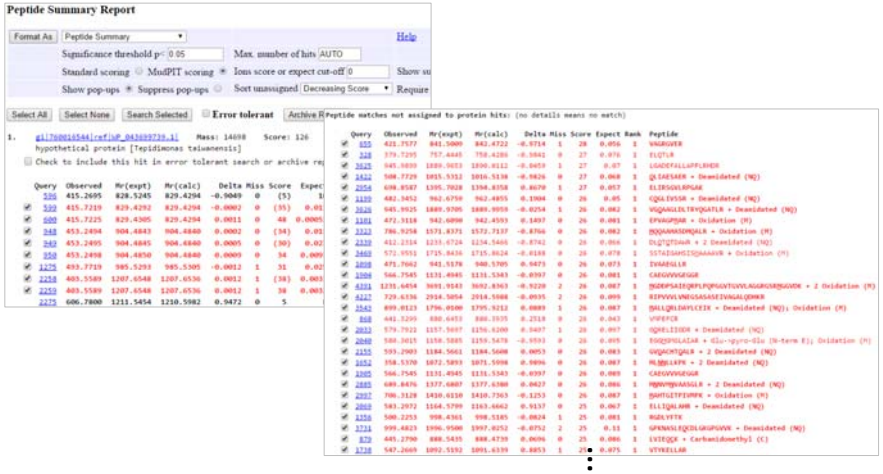


Lysis buffer, 8M urea

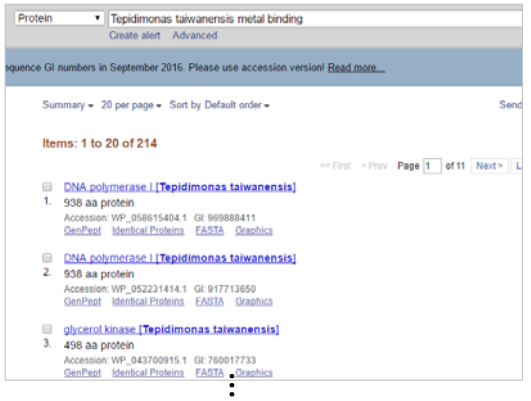
Lysis buffer, 7M urea+ 2M thiourea

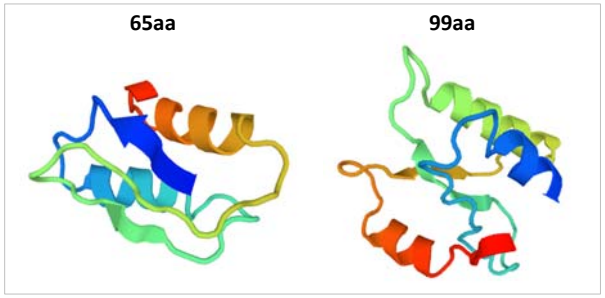
Mascot results

Mascot results



Search NCBI for the hypothetical proteins of "Tepidimonas taiwanensis metal binding".





The partial sequences of the proteins (65aa and 99aa) correspond with both Mascot non-hint data.

WP_058616432 (65 aa)

mhhvftvdgm scghcvkait qavraldpqa qvrvdlderr vevesdrsrv aladairdeg ytvrd

WP_052231443 (99 aa)

mrltpehval ikqvvaeqag tdaqvwlfgs raddtarggd vdllvqlprp iddpapcaar

iagrisramq grkvdvvmla pnlrrlsihe ealrsgvll

The corresponding sequences of **Approach A** were shown in yellow highlight and box.
Approach B were shown in color words.

Q & A

- What's the importance of enzyme purity?
- When will you used enzyme?
- Where can you obtain the enzyme?
- Who is the pioneer in enzyme study?
- How to use the proteomics and proteome?
- In vivo and in vitro are two central concepts of biologists approach. What' your opinion and recognition to both approaches?
- How to establish the new technology for enzymatic analysis?