Aula 11: 15/12/20 e 05/01/21

Estabilização de enzimas em solventes orgânicos e meios bifásicos

vídeo #1

1.1 Introduction

Any exponents of classical organic chemistry will probably hesitate to consider a biochemical solution for one of their synthetic problems. This would be due, very often, to the fact, that biological systems would have to be handled. Where the growth and maintainance of whole microorganisms is concerned, such hesitation is probably justified. In order to save endless frustrations, close collaboration with a biochemist is highly recommended to set up and use fermentation systems [1, 2]. On the other hand, isolated enzymes (which may be obtained increasingly easily from commercial sources either in a crude or partially purified form) can be handled like any other chemical catalyst [3]. Due to the enormous complexity of biochemical reactions compared to the repertoire of classical organic reactions, it follows that most of the methods described will have a strong empirical aspect. This 'black box' approach may not entirely satisfy the scientific purists, but as organic chemists are rather prone to be pragmatists, they may accept that the understanding of a biochemical reaction mechanism is not a conditio sine qua non for the success of a biotransformation [4]. In other words, a lack of understanding of biochemical reactions should never deter us from using them if their usefulness has been established. Notwithstanding, it is undoubtedly an advantage to have an acquaintance with basic biochemistry, and with enzymology in particular.

1.2 Common Prejudices Against Enzymes

If one uses enzymes for the transformation of non-natural organic compounds, the following prejudices are frequently encountered:

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- *'Enzymes are sensitive'.*

This is certainly true for most enzymes if one thinks of boiling them in water, but that also holds for most organic reagents, e.g. butyl lithium. If certain precautions are met, enzymes can be remarkably stable. Some candidates can even tolerate hostile environments such as temperatures greater than 100 °C and pressures beyond several hundred bar [5-7].

- *'Enzymes are expensive'.*

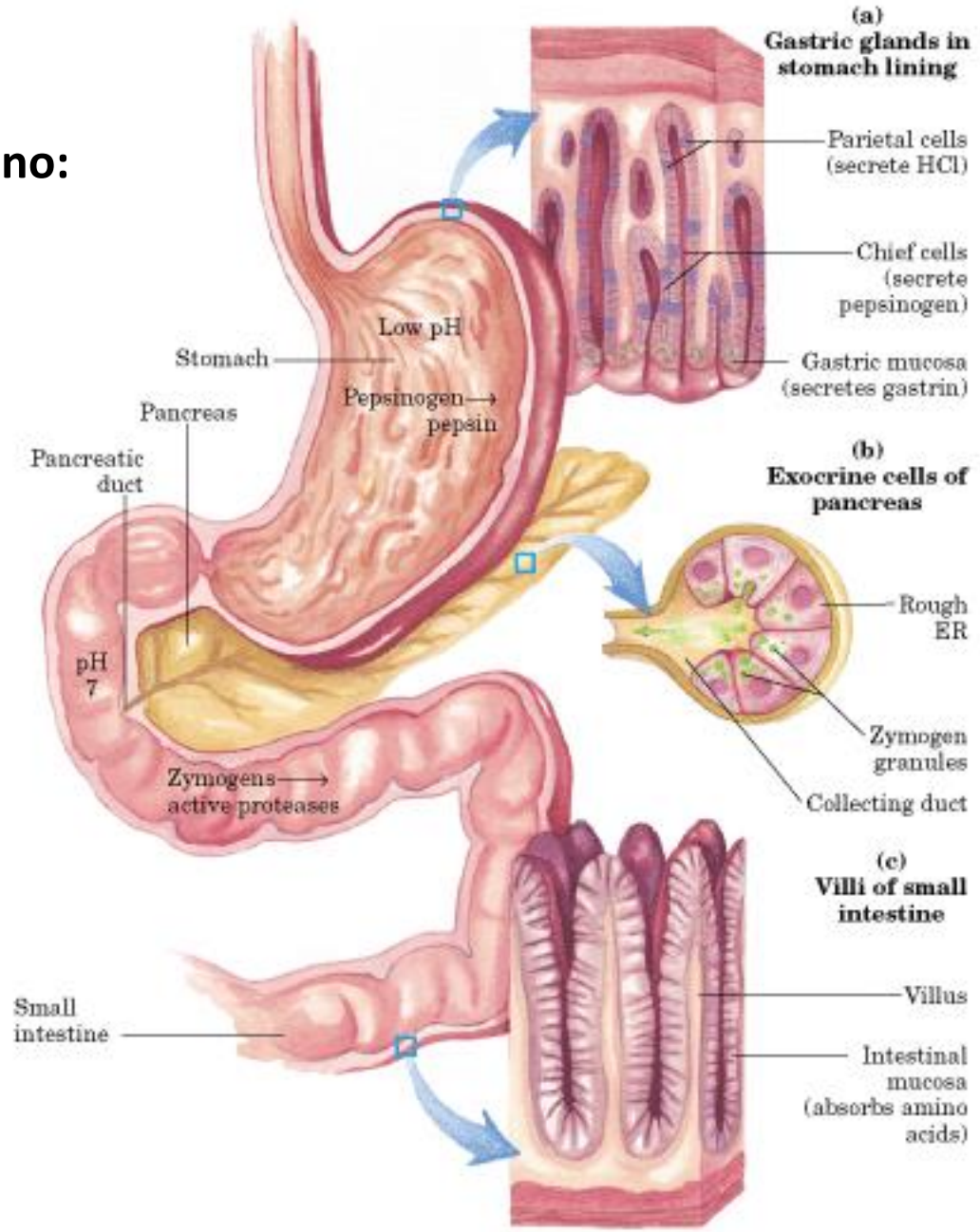
Some are, but others can be very cheap if they are produced on a reasonable scale. Considering the higher catalytic power of enzymes compared to chemical catalysts, the overall efficiency of a process may be better even if a rather expensive enzyme is required. Moreover, enzymes can be re-used if they are immobilized. It should be emphasized that for most chemical reactions relatively crude and thus reasonably priced enzyme preparations are adequate.

- *'Enzymes are only active on their natural substrates'.*

This statement is certainly true for some enzymes, but it is definitely false for the majority of them. Much of the early research on biotransformations was impeded by a tacitly accepted dogma of traditional biochemistry which stated that 'enzymes are Nature´s own catalysts developed during evolution for the regulation of metabolic pathways'. This narrow definition implied that man-made organic compounds cannot be regarded as substrates. Once this scholastic problem was surmounted [8], it turned out that the fact that Nature has developed its own peculiar catalysts over 3 x 10^9 years does not necessarily imply that they are designed to work only on their natural target molecules. Research during the past decade has shown that the substrate tolerance of many enzymes is much wider than previously believed and that numerous biocatalysts are capable of accepting non-natural substrates of an unrelated structural type by often exhibiting the same high specificities as for the natural counterparts. It seems to be a general trend, that the more complex the enzyme´s mechanism, the narrower the limit for the acceptability of 'foreign' substrates. It is a remarkable paradox that many enzymes display high specificities for a specific type of reaction while accepting a wide variety of substrate structures. After all, there are many enzymes whose natural substrates - if there are any - are unknown.

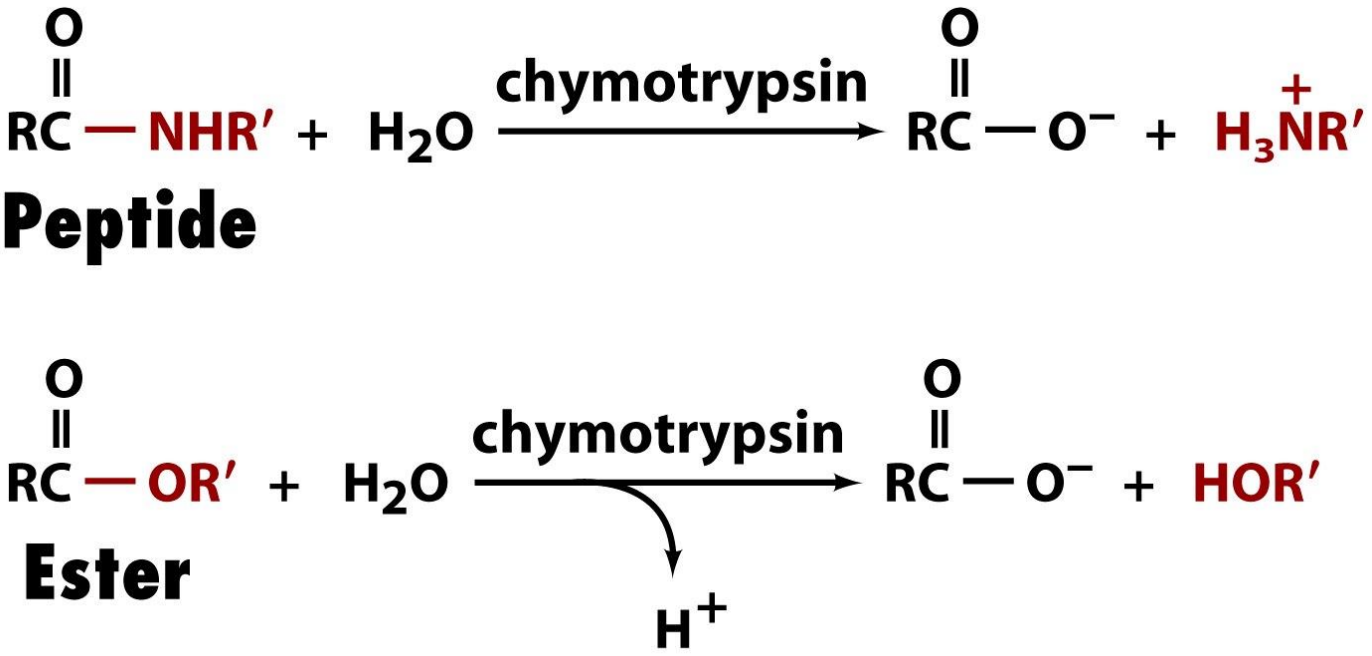
One of the major barriers to the use of enzymes in industrial biotechnology is their insufficient stability under processing conditions (*Stepankova et al., 2013*)

EX 1:
Sistema digestório humano:
enzimas <u>em água</u>



Ex 2: Enzima pancreática da classe das serino-proteases ou peptidase

Atividades amidásica e esterásica



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- **Água favorece reações de hidrólise**
- **Água tem alto ponto de ebulição e baixo calor de vaporização**

(*solvente estável a 36-37°C ou superior*)

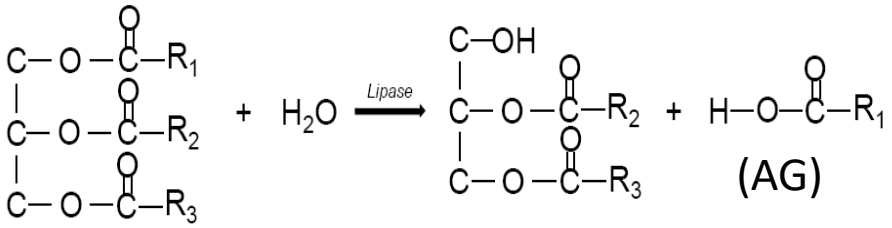
- **Água participa na determinação da estrutura proteica e em processos que levam à desnaturação** *(até quanto poderíamos reduzir?)*

De fato,
na natureza enzimas e complexos enzimáticos atuam em água, mas também em outros ambientes *(mais hidrofóbicos e bifásicos):*
(i) Em interfaces de solução aquosa/solução orgânica
(ii) Ligadas a membranas celulares

Ex (i): Lipase pancreática - atividade esterásica sobre substratos lipídicos

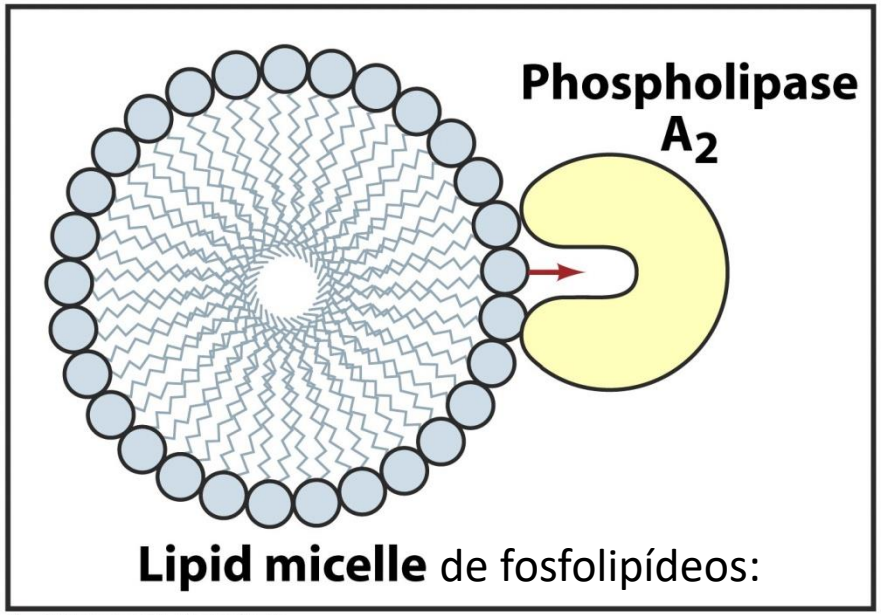
Reações químicas :

Hydrolysis de TAG (gordura neutra):



Methanolysis de AG:

Modelo esquemático da interação entre Enzima e Substrato → **Enzima na interface**



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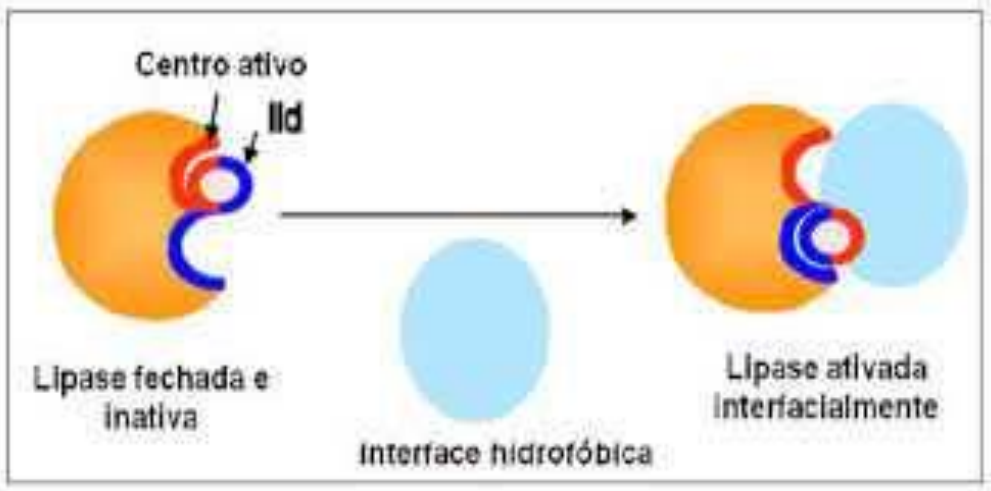
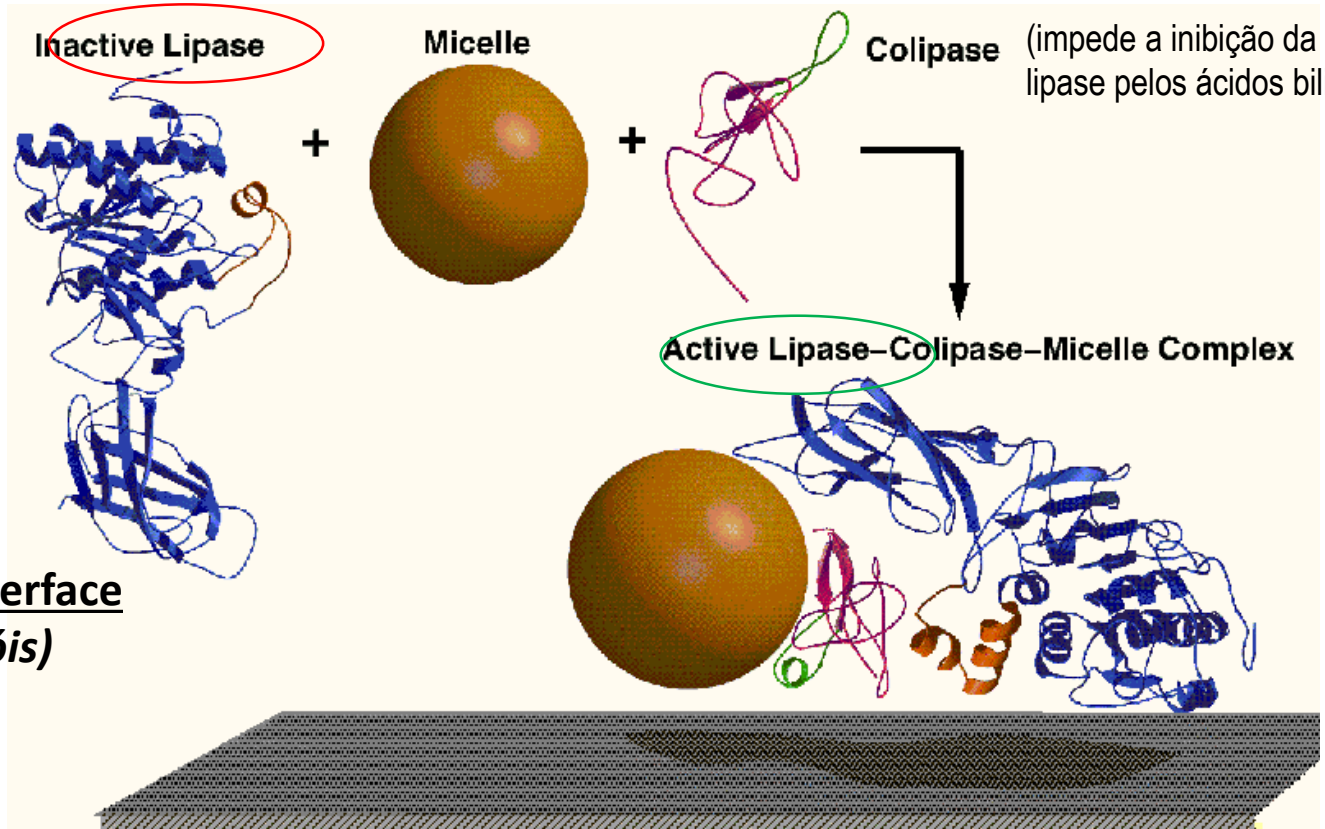
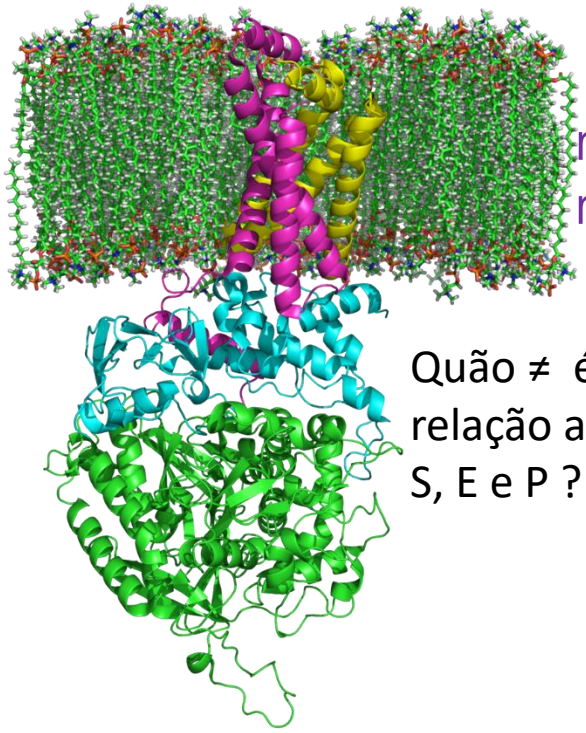
Ativação interfacial de lipase





Lipase pancreática
<u>em presença de interface</u>
(água/triacilgliceróis)

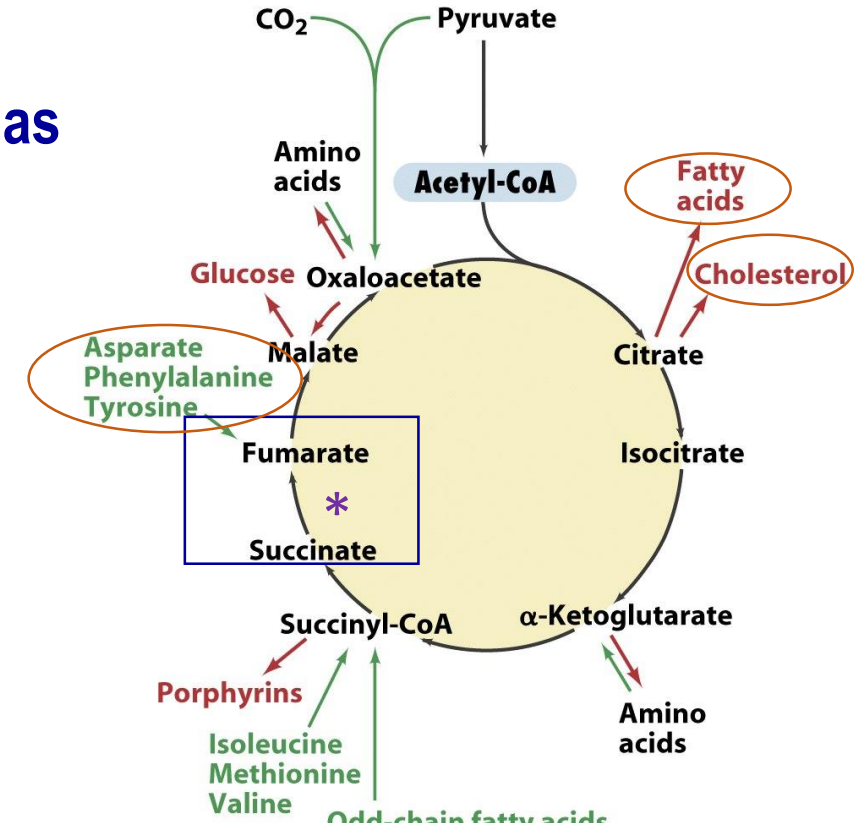
Ex (ii): Enzimas ligadas a membranas celulares



membrana mitocondrial

matriz mitocondrial

Quão ≠ é esse meio em relação ao aquoso para S, E e P ?



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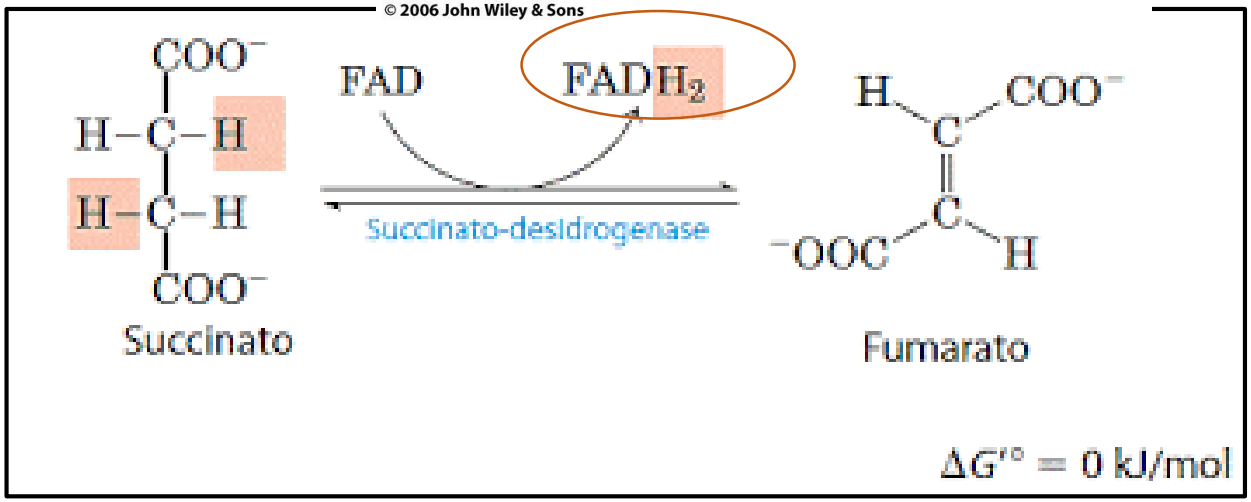


Bis(2-ethylhexyl)
sulfosuccinate sodium salt

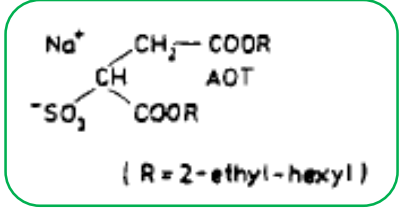


Figure 1. Schematic representation of the solubilization of enzymes in reverse micelles (cross section) and of the structure of the surfactant AOT used in our studies. Particularly at small w_o values (w_o = $[H_2O]/[AOT]$) the uptake o
ration of the material present

Reações enzimáticas *in vitro* em micelas invertidas *(reversas)*

- Partição de S e P das reações catalisadas

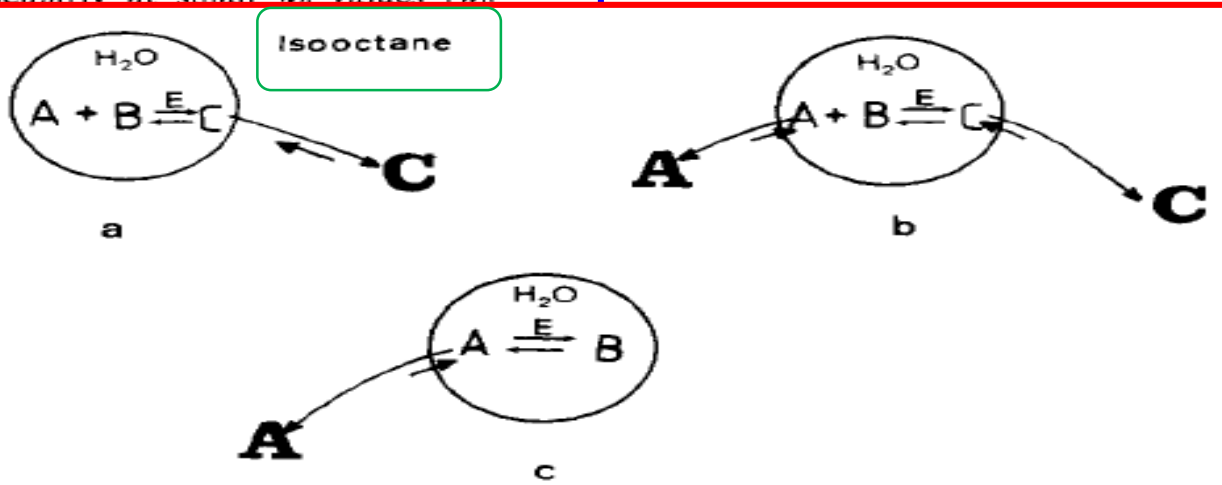
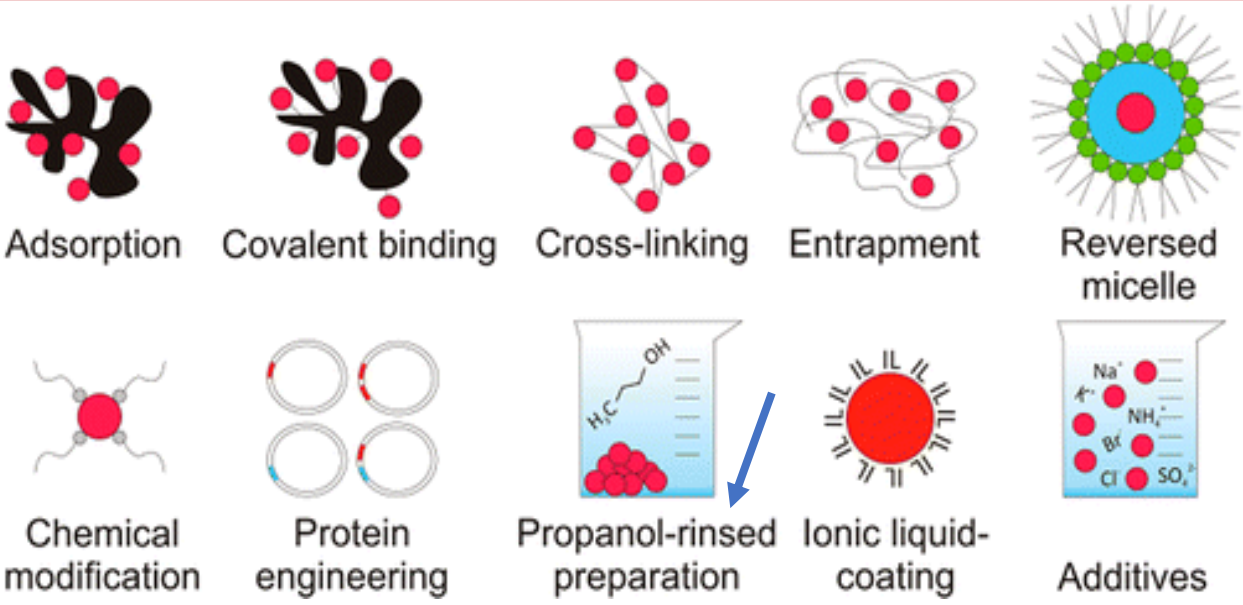


Figure 2. Three examples of compartimentalization of reactants in reverse micelles in enzymatic reactions. In the first case (a) the two reagents A and B are preferentially soluble in water and the product C in hydrocarbon. The second case (b) represents a different reaction, where one of the two reagents (A) is also soluble in hydrocarbon. Finally (c), the case of a quite different reaction is schematized, where an overwhelming hydrocarbon-soluble compound A yields, upon enzymatic cleavage, a water soluble product B which would remain entrapped in the water pools.

Mas... e enzimas em solventes orgânicos (SO) e em outros sistemas bifásicos?

Aulas 9 e 10 desta disciplina: *melhoria de enzimas*
Modificação, Mutação, Imobilização de enzimas / NBCs



Stepankova *et al.* (2013) **Strategies for Stabilization of Enzymes in Organic Solvents**, *ACS Catalysis*, *3* (12), 2823–36

The **use of organic solvent systems instead of aqueous media for enzymatic reactions offers numerous advantages**, such as increased solubility of hydrophobic substrates or suppression of water-dependent side reactions….

However, **organic solvents often inactivate enzymes → Aulas de BQ: E precipitam e denaturam**

Industry and academia have devoted considerable effort into developing **effective strategies to enhance the lifetime of enzymes in the presence of organic solvents**.

Vá para vídeo #2

Em meio contendo apenas solventes orgânicos?

retenção da atividade de quimotripsina em hexano

↓

conformação nativa não foi drastica/e alterada no meio mais hidrofóbico:

comprovado por:
N^{15}-RMN, C^{13}-RMN,
cristalografia de raios X

Proposição:

Em SO: rigidez estrutural

H_2O = "lubrificante"

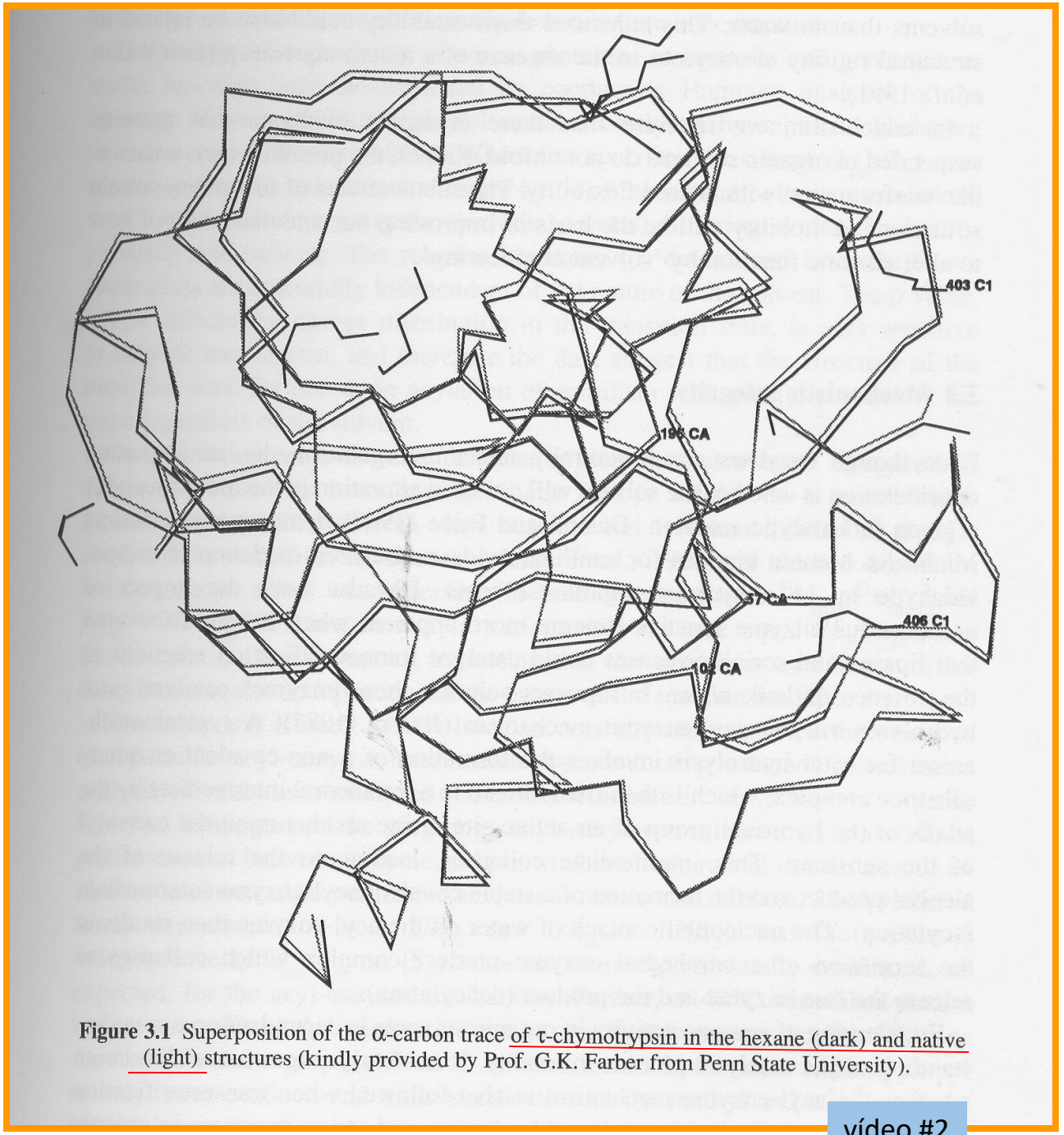


Figure 3.1 Superposition of the α-carbon trace of τ-chymotrypsin in the hexane (dark) and native (light) structures (kindly provided by Prof. G.K. Farber from Penn State University).

vídeo #2

E pkas e pH ótimo; afetados pela alteração da constante dielétrica do meio?

- alteração de pKas de S, mas não de E (*água residual* e "*pH memory em > 98% SO*")

→ estrutura 3D mantida → função catalítica também

solvatação e ionização diferentes de S → **alteração de Km**

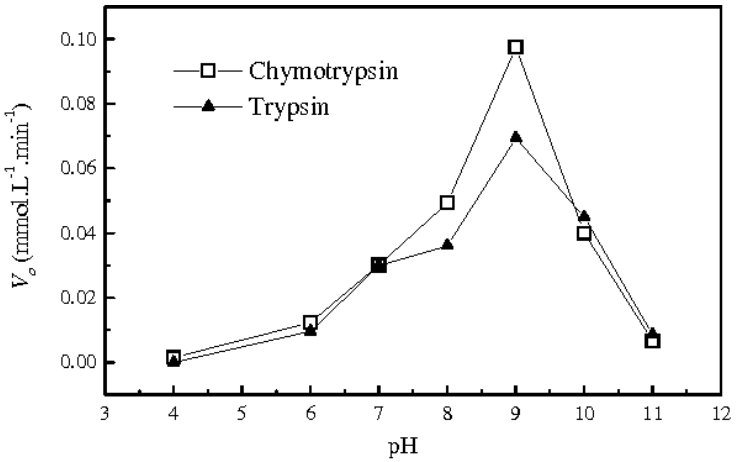


FIGURE 2 – Optimum pH of trypsin and chymotrypsin

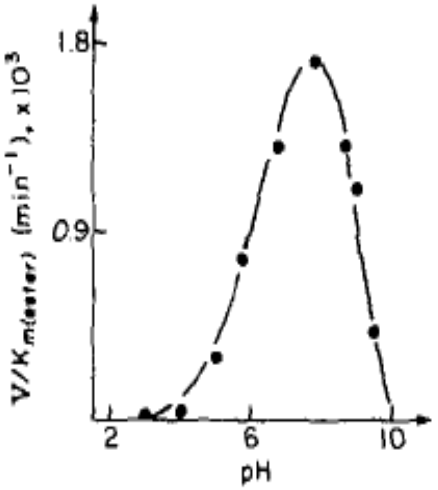


FIG. 1. **The dependence of the enzymatic activity of chymotrypsin in octane on the pH of the aqueous solution from which the enzyme was lyophilized.** Chymotrypsin (5 mg/ml) was lyophilized from aqueous solutions of different pH containing 0.25% *N*-acetyl-L-phenylalanine (to activate the enzyme in octane, see text). Samples of the enzyme (1 mg) were then added to 1 ml of octane containing the substrates *N*-acetyl-L-phenylalanine ethyl ester and *n*-propanol, the suspensions were shaken at 20 °C and 250 rpm, and the values of $V/K_{m(ester)}$ were determined as cotangents in the double reciprocal coordinates as outlined in the text.

O uso de <u>solventes orgânicos ao invés da água</u> em reações catalisadas por enzimas oferece vantagens:

- ❖ Maior solubilidade de determinados substratos;
- ❖ Supressão de reações 2ªs. água-dependentes

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Qual é a necessidade mínima de H_2O para manutenção da atividade catalítica?

R = É enzima-dependente:

1) α-Quimotripsina: 50 moléculas /molécula de enzima
(menos do que o necessário para monocamada de água ao redor dela)

2) Subtilisina : traços de água

3) Lipases: traços de água

4) Polifenol-oxidase: $3{,}5 \times 10^7$ moléculas de água
Alcool desidrogenase, alcool oxidase: similares

Estudos mostram: ***Em soluções aquosas essa necessidade é irrelevante.***
Em meios orgânicos essa necessidade é relevante.

Conteúdo de H_2O na reação enzimática em meio de SO é somatória de:

"bound water" = pequena fração de água que fica estreitamente ligada à superfície da enzima isolada = crucial para a manutenção da estrutura ativa = "água estrutural" = "água residual"

"bulk water" = água presente no meio reacional

Meio orgânico (SO) *X* quanto de H_2O residual?

TABLE II

The amount of water on chymotrypsin following its incubation in different organic solvents

pH-adjusted and activated with *N*-acetyl-L-phenylalanine (see text) samples of chymotrypsin (50 mg) were placed in organic solvents (50 ml), and the suspensions were shaken at 20 °C for 1 h. The enzyme was then removed by centrifugation, and the amount of bound water was determined by the Fischer method. All organic solvents contained less than 0.02% (v/v) water (the sensitivity of our detection method). Each water content value presented in the table is a result of three independent measurements.

Solvent	Residual water content
	% (w/w)
Octane	2.5 ± 0.10
Toluene	2.3 ± 0.09
Tetrahydrofuran	1.6 ± 0.11
Acetone	1.2 ± 0.08
Pyridine	1.0 ± 0.08

Conclusão:

SO - polar : E com + H_2O residual

SO + polar : E com - H_2O residual

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Como mediu?

The Water Determination Test (Karl Fischer Method) is designed to determine water content in substances, utilizing the quantitative reaction of water with iodine and sulfur dioxide in the presence of a lower alcohol such as methanol and an organic base such as pyridine, as shown in the following formulae:

$$H_2O + I_2 + SO_2 + 3\ C_5H_5N \rightarrow 2(C_5H_5N{+}H)I^- + C_5H_5N \cdot SO_3 + CH_3OH \rightarrow (C_5H_5N{+}H)OSO_2 \cdot OCH_3.$$

There are two determination methods different in iodine-providing principle: the volumetric titration method and the coulometric titration method.

Convencionalmente,
considera-se para uma reação catalisada por Enzima:

A + B → C

pH ótimo → escolha do tampão
temperatura
tipo de agitação
[A], [B], [**E**]
forma de E: bruta/purificada, mas solúvel

↓

Condições reacionais

↓

Velocidade de reação
Rendimento da reação em determinado tempo

Mas, são fatores igualmente importantes:

1) **solubilidade de A, B e C no meio reacional → busca de outros meios**
2) **quantidade de água no meio reacional nesses meios**
3) **estrutura e propriedades da enzima em outros meios reacionais**
4) **outras formas da enzima: p. ex. insolúvel no meio reacional**
5) **produtividade**

1) Solubilidade de S ou reagentes de natureza orgânica?

Tampões + SO ou apenas SO (álcoois, AcOEt, Hexano, octano, tolueno)

2) Quanto de água existe de fato nessas reações ?

Informação correta → conclusões corretas sobre os fatores que afetam a reprodutibilidade das reações

Pode afetar os resultados

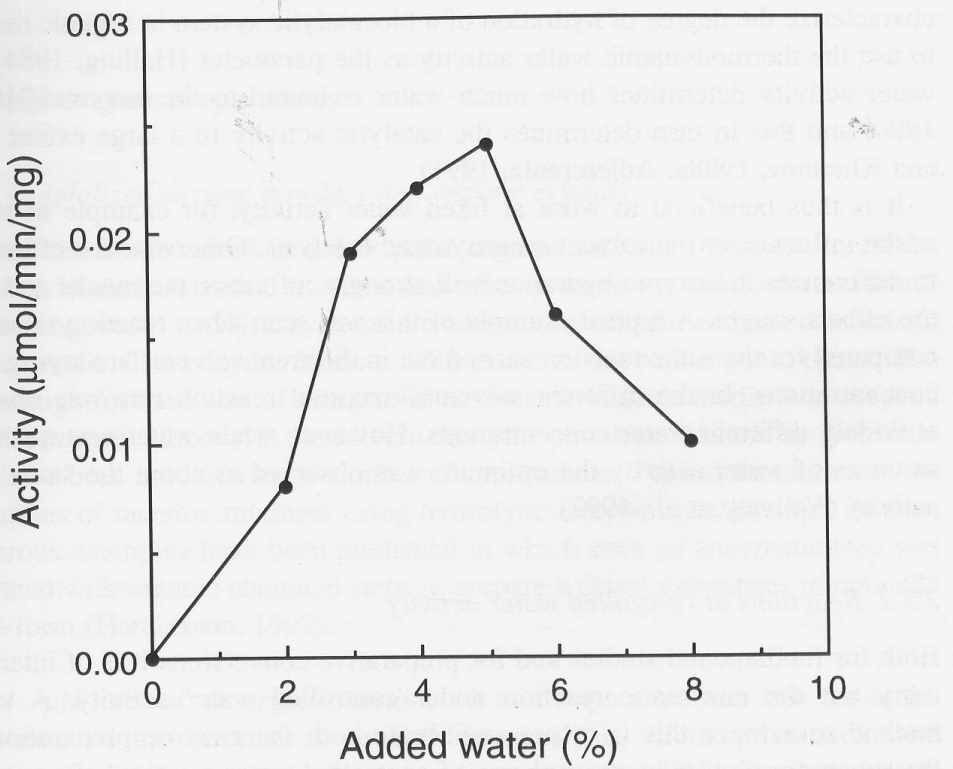

Figure 2.3 Catalytic activity of α-chymotrypsin in ethyl acetate with various amounts of water added. The enzyme was deposited on Chromosorb W/AW and catalyzed the esterification of *N*-acetyl-L-phenylalanine with ethanol. The solubility of water in the reaction medium was about 4.7%. Data from Wehtje *et al.* (1993b).

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2) E no meios reacionais, quais são fontes de água para reações catalisadas por enzimas?

- **Enzima** *(estrutural ou residual)*
- **Reagentes ou substratos** (compostos orgânicos; inclusive polímeros)
- **Sais** *(tampões)*
- **Solventes orgânicos** *(depende da polaridade)*
- **Ambiente** *(sistemas reacionais abertos)*
- **Geração de água** *(um dos produtos)*

2) Ex: Ac-Phe-OCH_2-CH_3 + R-OH → Ac-Phe-O-R + CH_2-CH_3 –OH (transesterificação)

subtilisina

aditivos (≠ % água)

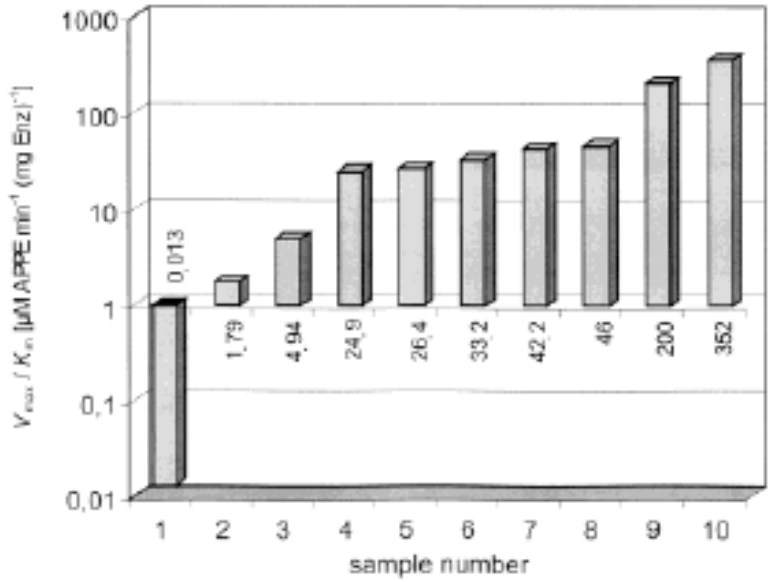

Figure 6. The progressive improvement of V_{max}/K_m for the transesterification of *N*-acetyl-phenylalanine ethyl ester (APPEE) in hexane by subtilisin activated by various combinations of additives (according to ref 157 with permission from John Wiley & Sons Inc. Copyright 2001). (1) salt-free enzyme, (2) PEG (MW = 2000), (3) 49% KCl/49% PEG, (4) KCl, (5) 49% $NaHCO_3$/49% PEG, (6) $NaHCO_3$, (7) 31.3% $NaHCO_3$/66.7% KCl, (8) $NaCH_3COO$, (9) 24.1% $NaCH_3COO$/73.9% $NaHCO_3$, (10) 24.1% $NaCH_3COO$/73.9% $NaHCO_3$ containing 0.8% water. In all cases, the total additive content was 98%.

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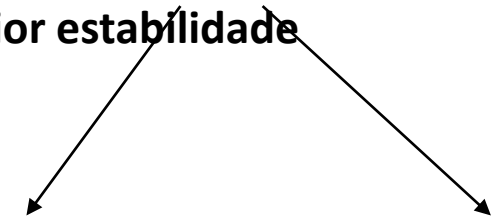
3) Estabilidade de E:

água e SO >98%

+ solvente; – água:

menor desnaturação

maior estabilidade



Vidas médias de **lipase pancreática suína**, **Ribonuclease** e **α-quimotripsina :**

em SO a 100°C → horas

em H_2O a 100°C → segundos

Provável:

Manutenção/alteração benéfica de estrutura

Proteólise diminuída

(menor interação com proteases de microorganismos contaminantes)

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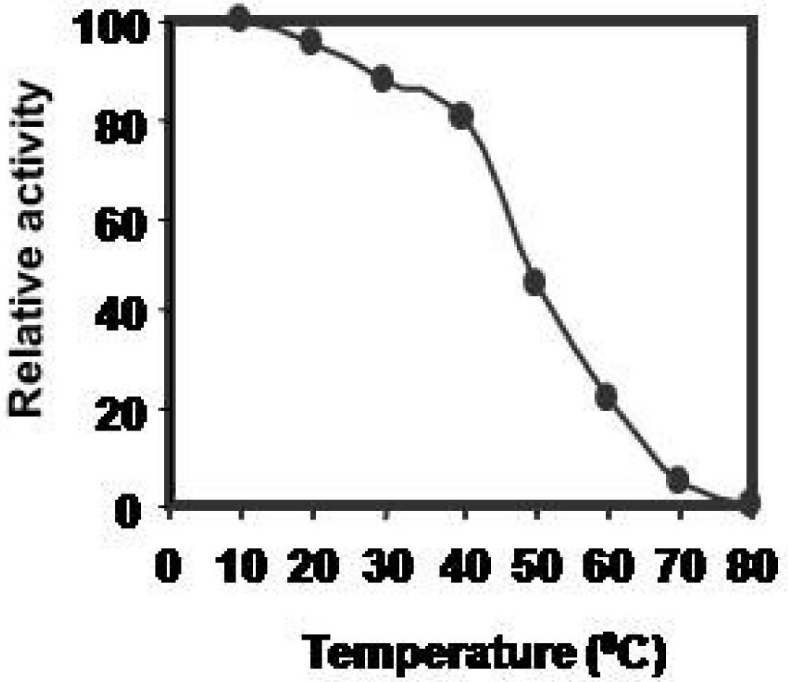
Table 3. Half-Life of *Pseudomonas aeruginosa* PST-01 Protease and Thermolysin in Organic Media[a]

	half-life (day)	
solvent	PST-01 protease	thermolysin
ethylene glycol	>100	>50
1,4-butandiol	>100	4.4
1,5-pentandiol	>100	1.7
ethanol	>100	3.0
1-hexanol	>50	18.2
methanol	>50	4.6
DMSO	>50	2.6
2-propanol	>50	1.2
triethylene glycol	>50	5.1
tert-butanol	>50	0.8
1-heptanol	>50	13.1
DMF	25.3	0.9
1-octanol	24.2	n.t.
1-butanol	24.2	0.9
acetone	23.1	0.7
1-decanol	19.4	n.t.
1,4-dioxan	17.7	0.8
toluene	12.0	22.5
benzene	7.8	n.t.
n-heptane	4.8	n.t.
p-xylene	4.4	n.t.
n-hexane	3.8	n.t.
n-decane	2.4	n.t.
cyclohexane	2.3	n.t.
aqueous medium	9.7	10.8

[a] According to ref 168b with permission from the Society of Biosciences and Bioengineering. Copyright 1999. To solutions of 3 mL containing the appropriate protease 1 mL of organic solvent was added and the residual activities were measured for 15 days. The half-lives reported were calculated from the exponential regression curves. n.t., not tested.

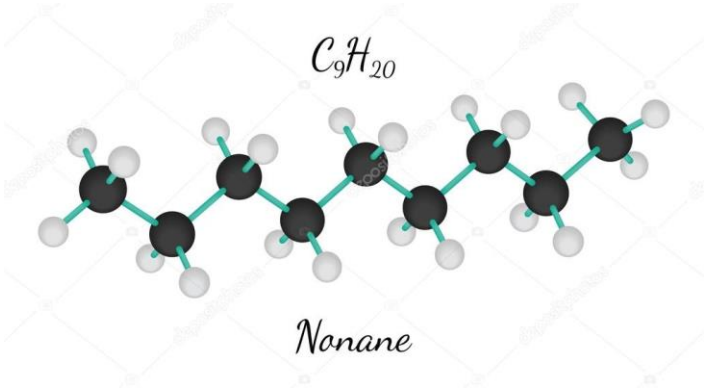
3) Denaturação térmica X água e SO >98%



Desenovelamento (desnaturação térmica) da ribonuclease:

124°C em nonano

61°C em H_2O



3) Outros exemplos

Table 4.1 Stability of enzymes in non-aqueous vs. aqueous media

SO ≥ 90 %

Enzyme	Conditions	Thermal property	References
PPL	tributyrin (lipídeo)	$t_{1/2}$ < 26 h	Zaks and Klibanov (1984)
	aqueous, pH 7.0	$t_{1/2}$ < 2 min	
Candida lipase	tributyrin/heptanol	$t_{1/2}$ 1.5 h	Zaks and Klibanov (1984)
	aqueous pH 7.0	$t_{1/2}$ < 2 min	
Chymotrypsin	octane, 100°C	$t_{1/2}$ 80 min	Zaks and Klibanov (1988)
	aqueous, pH 8.0, 55°C	$t_{1/2}$ 15 min	Martinek *et al.* (1977)
Subtilisin	octane, 110°C	$t_{1/2}$ 80 min	Russell and Klibanov (1988)
Lysozyme	cyclohexane, 110°C	$t_{1/2}$ 140 h	Ahern and Klibanov (1986)
	aqueous	$t_{1/2}$ < 10 min	
Ribonuclease	nonane, 110°C, 6 h	95% activity remains	Volkin and Klibanov (1990)
	aqueous, pH 8.0, 90°C	$t_{1/2}$ < 10 min	
F_1-ATPase	toluene, 70°C	$t_{1/2}$ > 24 h	Garza-Ramos *et al.* (1989)
	aqueous, 70°C	$t_{1/2}$ < 10 min	
Alcohol dehydrogenase	heptane, 55°C	$t_{1/2}$ > 50 days	Kaul and Mattiasson (1993)
Hind III	heptane, 55°C, 30 days	no loss of activity	Kaul and Mattiasson (1993)
Lipoprotein lipase	toluene, 90°C, 400 h	40% activity remains	Ottoline *et al.* (1992)
β-Glucosidase	2-propanol, 50°C, 30 h	80% activity remains	Tsitsimpikou *et al.* (1994)
Tyrosinase	chloroform, 50°C	$t_{1/2}$ 90 min	Yang and Robb (1993)
	aqueous solution, 50°C	$t_{1/2}$ 10 min	
Acid phosphatase	hexadecane, 80°C	$t_{1/2}$ 8 min	Toscano et al. (1990
	aqueous, 70°C	$t_{1/2}$ 1 min	
Cytochrome oxidase	toluene, 0.3% water	$t_{1/2}$ 4.0 h	Ayala *et al.* (1986)
	toluene, 1.3% water	$t_{1/2}$ 1.7 min	

K. Faber,
Biotransformation in Organic Chemisty
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4) Formas da enzima usada em reações contendo SO e/ou interfaces?

geralmente : isolada ou purificada em meio aquoso

Sólida:
- **Liofilizada** (+ usual)
- **Liofilizada com aditivos** (?)
- **Em cristais com ligações cruzadas** (glutaraldeído → CLEAs, CLECs)
- **Precipitada diretamente em SO**
- **Imobilizada**

Solubilizada
- **natural**
- **modificada de forma não covalente** (com surfactantes e polímeros orgânicos)
- **dentro de micelas reversas ou gotas de micro-emulsão**

5) Produtividade X Tipos de meios reacionais usados contendo SO?

1) Enzima dissolvida em soluções aquo-orgânicas monofásicas

Maioria dos solventes orgânicos polares

+ usados: DMSO, DMF, THF, dioxano, acetona, alcoois

(10-70% em vol.)

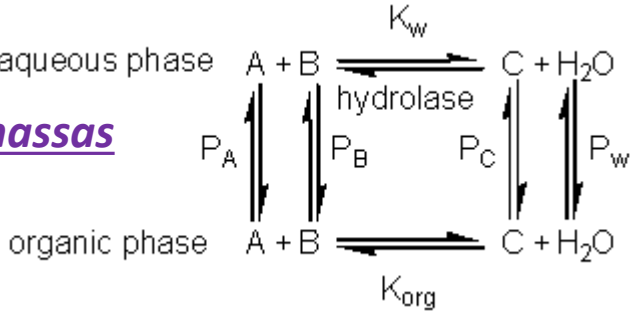
2) Enzima dissolvida em soluções aquo-orgânicas bifásicas

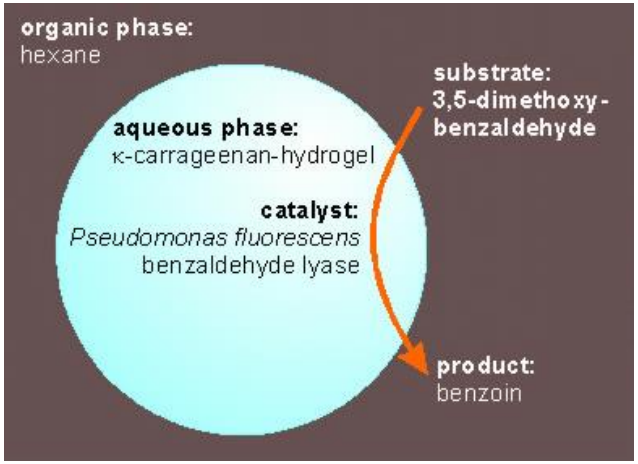
Solventes orgânicos apolares

Hidrocarbonetos, éteres, AcOEt, DCM; agitação vigorosa.

Partições de S e P

Transferência de massas





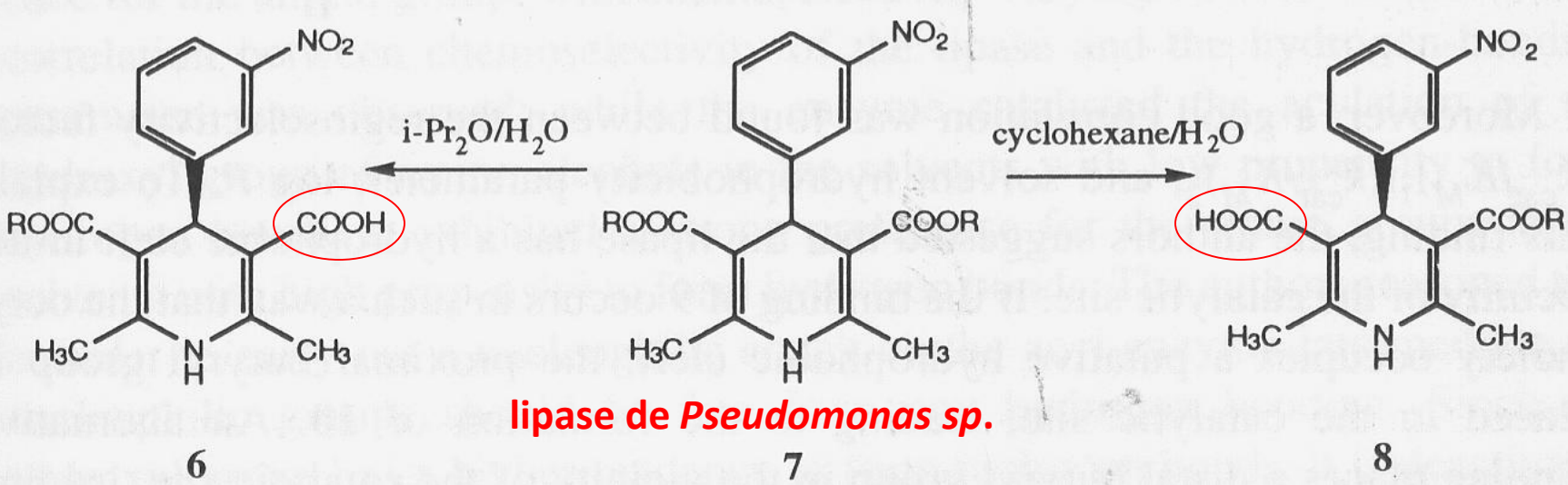
3) Enzima suspensa apenas em solvente orgânico

> 89% de SO : soluções monofásicas

Necessidade de água residual da enzima: mobilidade suficiente para formação de ES; agitação vigorosa.

Ex.: de alteração de propriedades: estereo-seletividade

Derivados de 1,4 dihidroxipiridina



(configuração S)

(configuração R)

Autores: interação entre solvente e enzima