Biotechnological applications of enzymes: proteases

Fields of application of proteases

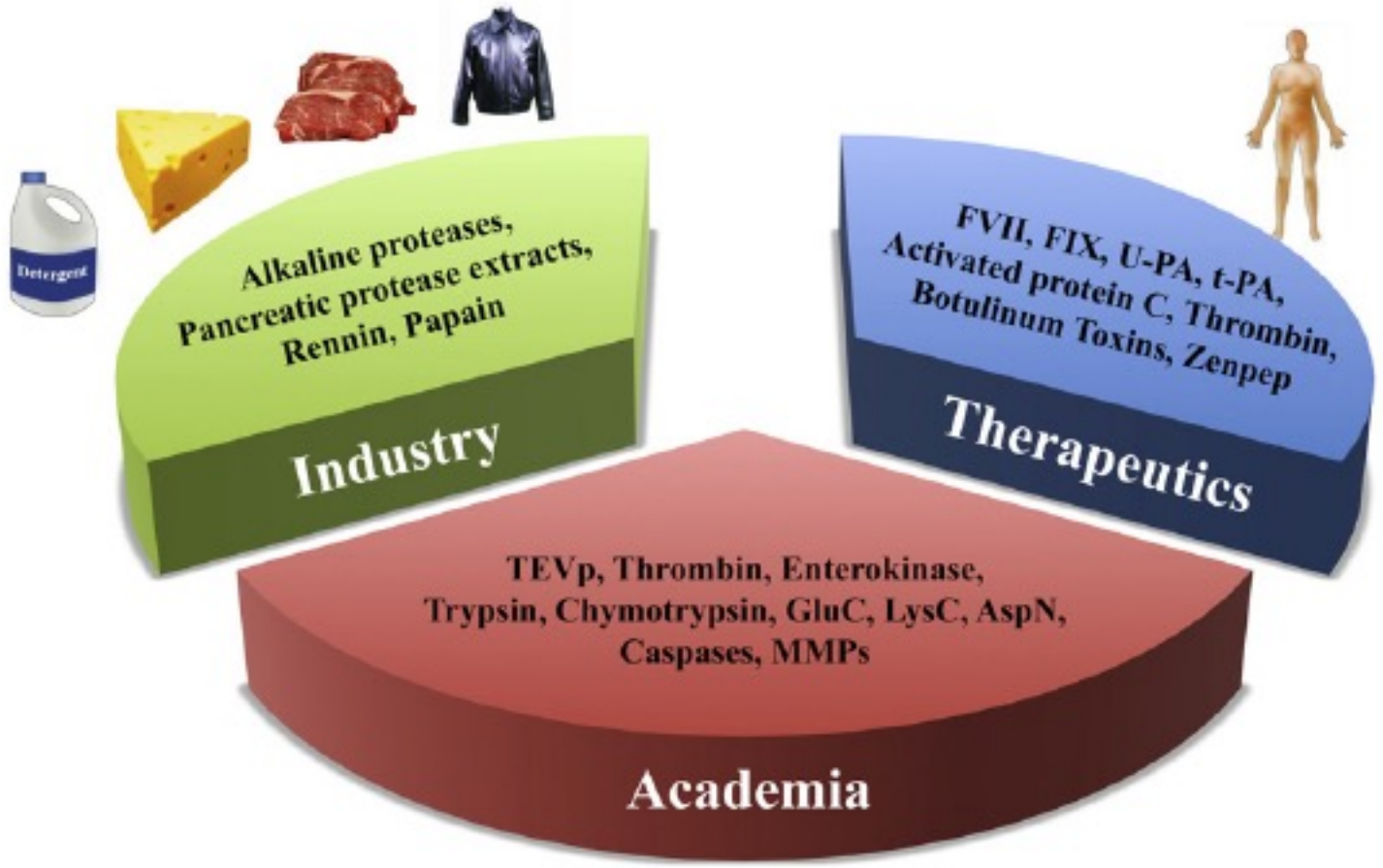


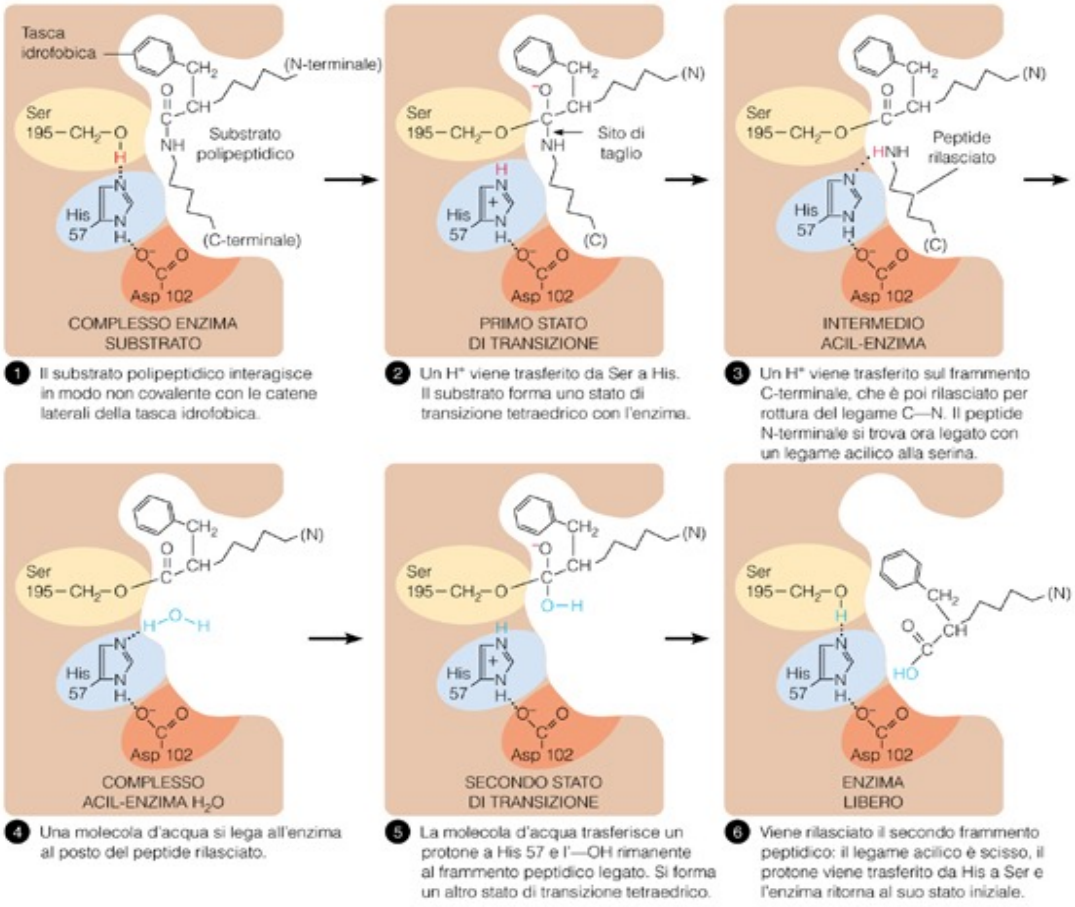
Fig. 1. An overview of protease applications.

Proteases catalyze the hydrolysis of peptide bonds

- Exopeptidases and endopeptidases
- Substrate specificity can be high or low
- 4 classes based on reaction mechanism:
  - Serine proteases (chymotripsin, trypsin, elastase, subtilisin)
  - Cysteine proteases (papain, caspase)
  - Acidic proteases (pepsin, chymosin, HIV protease)
  - Metalloproteases (carboxypeptidase A, thermolysin)

Many proteases possess also esterase activity

Reaction mechanism of serine proteases: covalent catalysis



Chymotripsin

Aspartic acid proteases: Pepsin

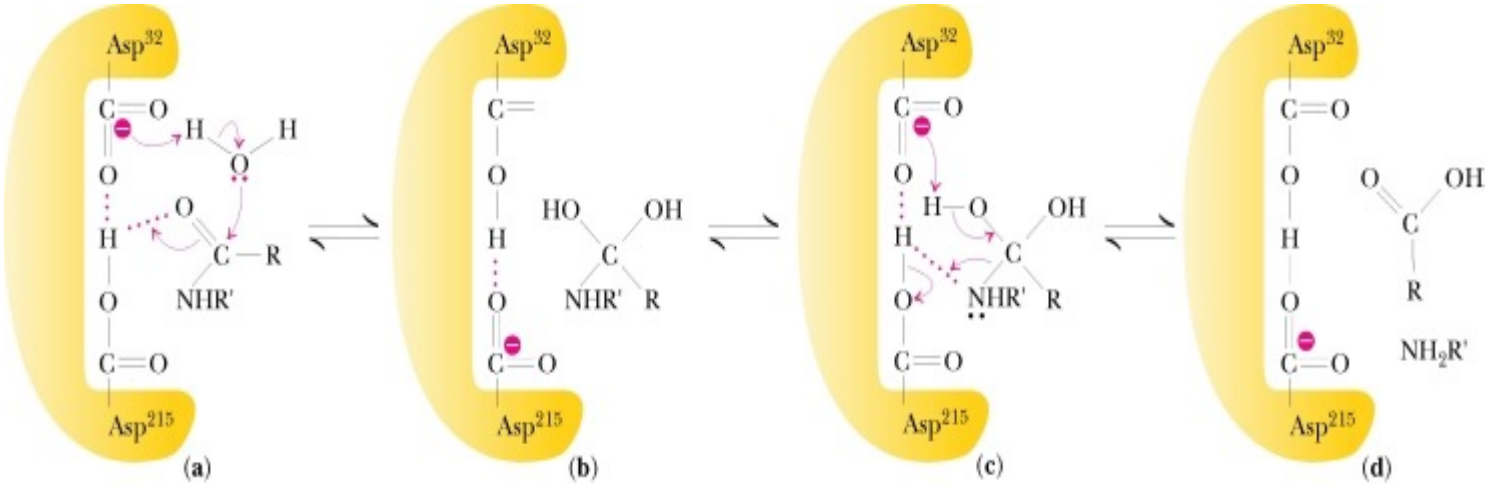
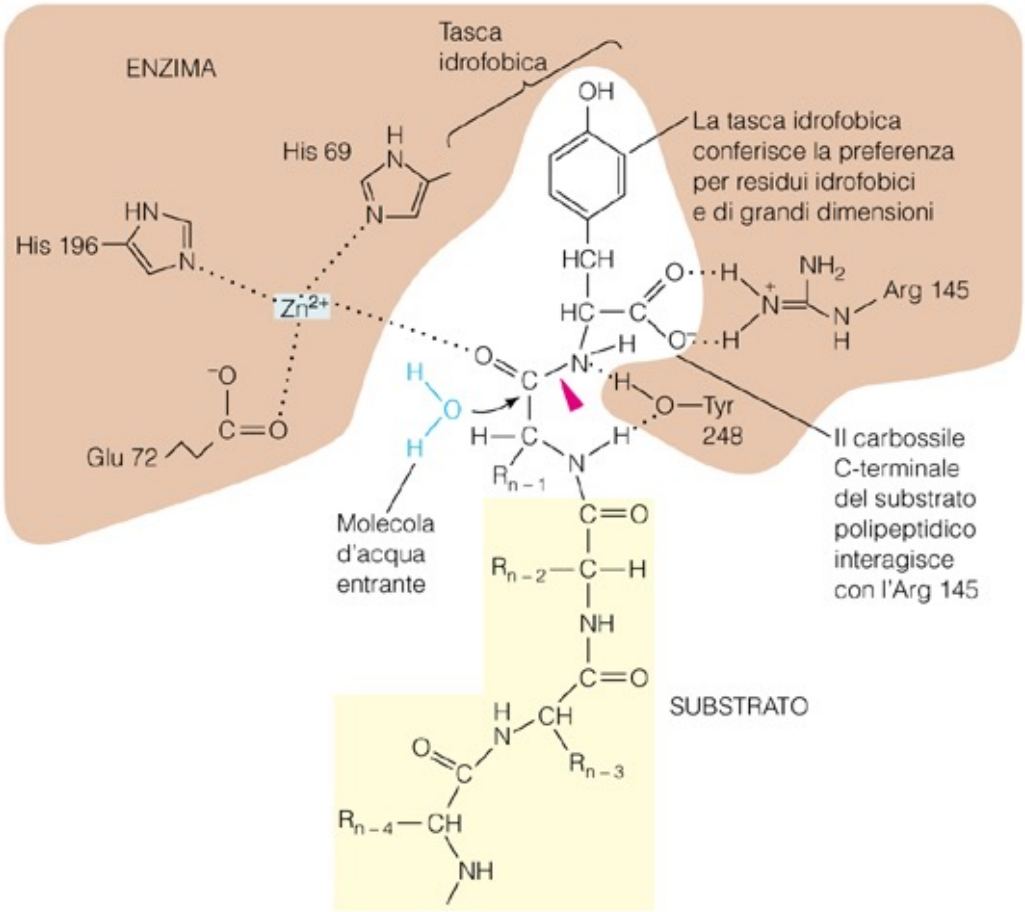


Figura 11.23 Un meccanismo per le aspartato proteasi. Nella prima fase, il trasferimento concertato di due protoni facilita l'attacco nucleofilico dell'acqua sul carbonio carbonilico del substrato. Nella terza fase, un residuo di aspartato (Asp^{32} nella pepsina) accetta un protone da uno dei gruppi ossidrilici dell'ammide diidrato mentre l'altro aspartato (Asp^{215}) cede un protone all'azoto dell'ammide che verrà rilasciata.

Metalloproteases: Carboxypeptidase A



Substrate specificity of different proteases

Table 1
Specificity of proteases[a]

Enzyme	Preferred cleavage site[b] N-terminal C-terminal
Serine proteases	
Trypsin	–Arg (or Lys)↓–Yaa–
Achromobacter protease	–Lys↓–Yaa–
Chymotrypsin, subtilisin	–Trp (or Tyr, Phe, Leu)↓–Yaa–
Elastase, α-lytic protease	–Ala (or Ser)↓–Yaa–
Proline-specific protease	–Pro↓–Yaa–
Staphylococcus V8 protease	–Asp (or Glu)↓–Yaa–
Carboxypeptidase Y	–Xaa↓–Yaa–
Thiol proteases	
Papain, *Streptococcus* protease	–Phe (or Val, Leu)-Xaa↓–Yaa–
Clostripain, cathepsin B	–Arg↓–Yaa–
Cathepsin C	H-X-Phe (or Tyr, Arg)↓–Yaa–
Metal proteases	
Thermolysin	–Xaa↓–Leu (or Phe)–
Myxobacter protease II	–Xaa↓–Lys–
Aspartic proteases	
Pepsin	–Phe (or Tyr, Leu)↓–Trp (or Phe, Tyr)–

[a]Data from Ref. 3.
[b]Xaa, various amino acid residues; Yaa, various amino acid residues, ester or amide.

Use of proteases as additives in detergents

Proteases used as additives in detergents must:

- Have low substrate specificity
- Have high activity both at low and high T
- Have high pH optimum
- Be resistant to other agents present in detergents (soap, metal chelators, oxidants etc.).

Subtilisins are serine proteases that possess these features

Table 1

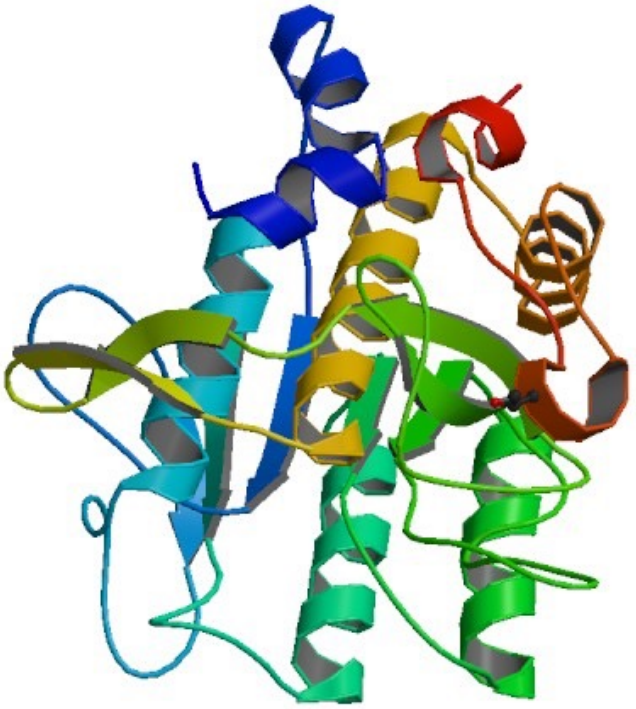
Subtilisin variants used in detergents.

Trade mark	Producer	Origin	WT/PE[c]	Production strain	Synonym
Alcalase®	Novozymes	*B. licheniformis*	WT	*B. licheniformis*	Subtilisin Carlsberg
FNA[a]	Genencor	*B. amyloliquefaciens*	PE	*B. subtilis*	
Savinase®	Novozymes	*B. clausii*	WT	*B. clausii*	Subtilisin 309
Purafect™	Genencor	*B. lentus*	WT	*B. subtilis*	
KAP[b]	Kao	*B. alkalophilus*	WT	*B. alkalophilus*	
Everlase™	Novozymes	*B. clausii*	PE	*B. clausii*	
Purafect OxP™	Genencor	*B. lentus*	PE	*B. subtilis*	
FN4[a]	Genencor	*B. lentus*	PE	*B. subtilis*	
BLAP S[b]	Henkel	*B. lentus*	PE	*B. licheniformis*	
BLAP X[b]	Henkel	*B. lentus*	PE	*B. licheniformis*	
Esperase®	Novozymes	*B. halodurans*	WT	*B. halodurans*	Subtilisin 147
Kannase™	Novozymes	*B. clausii*	PE	*B. clausii*	
Properase™	Genencor	*B. alkalophilus PB92*	PE	*B. alkaliphilus*	

[a]Exclusive molecules for specific customer.
[b]Exclusive molecules for captive use. The names of captive use products are often based on technical terms or acronyms.
[c]PE, protein engineered; WT, wild type.

Subtilisins

- Produced by *Bacillus* sp., they are extracellular alkaline proteases
- Serine proteases of about 27 kDa, with low substrate specificity and high stability
- In the EU > 1000 tons of pure subtilisin are produced and/or imported per year
- Used as additive in all types of detergents



- Subtilisin is one of the most engineered enzymes: over half of its 275 aminoacids have been targeted by mutagenesis!

Strategies to improve the properties of an enzyme

Which properties do you want to improve?

- Catalytic activity
- Substrate specificity
- New activities
- Stability
- Stability in 'exotic' environments (non-physiologic)

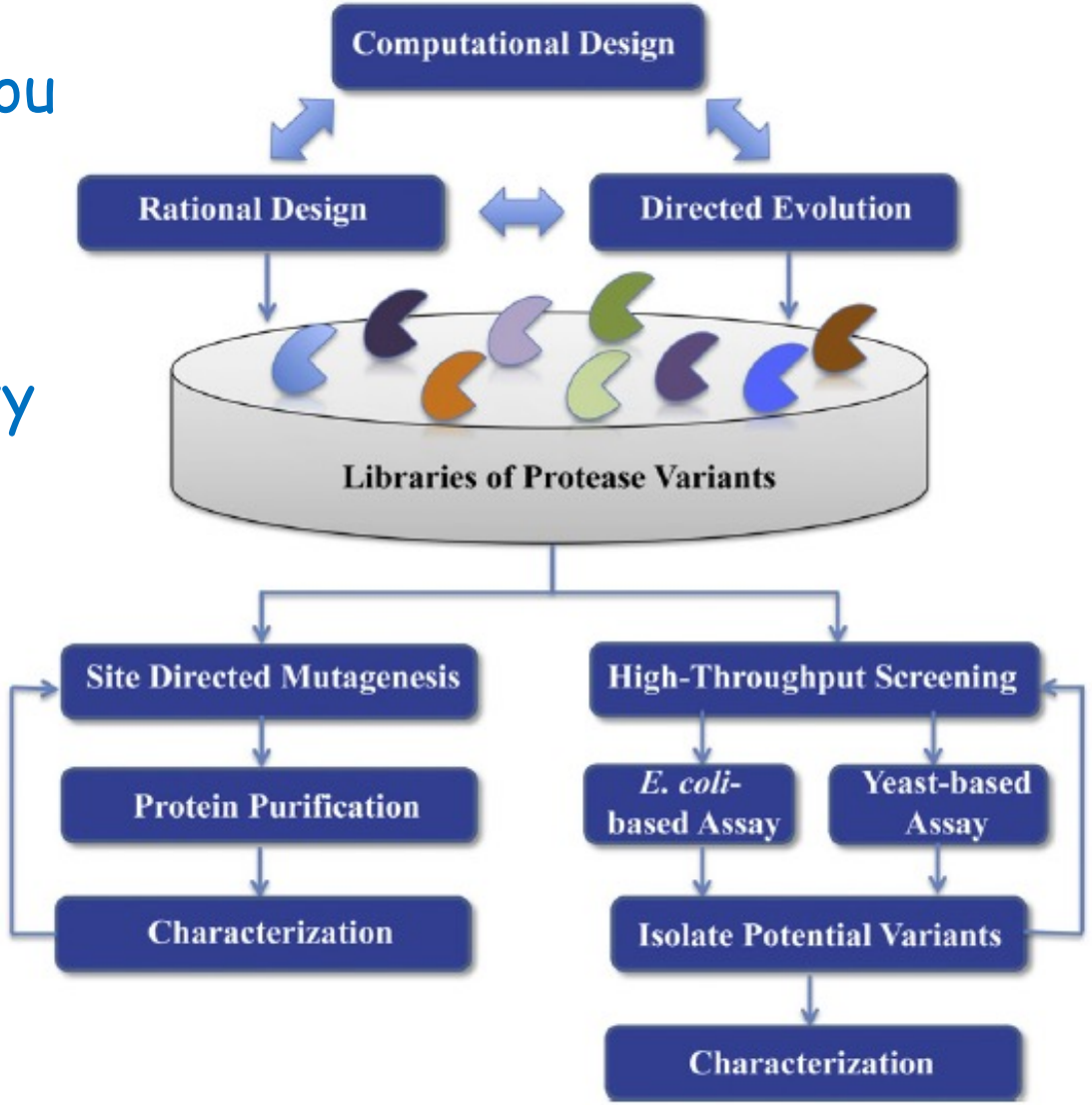


Fig. 2. Schematic diagram outlining the general approaches of protease engineering.

Use of proteases as additives in detergents.

Maxacal is a protease from *Bacillus lentus* that shows high activity at high pH and is thermostable.
It belongs to the subtilisin family.

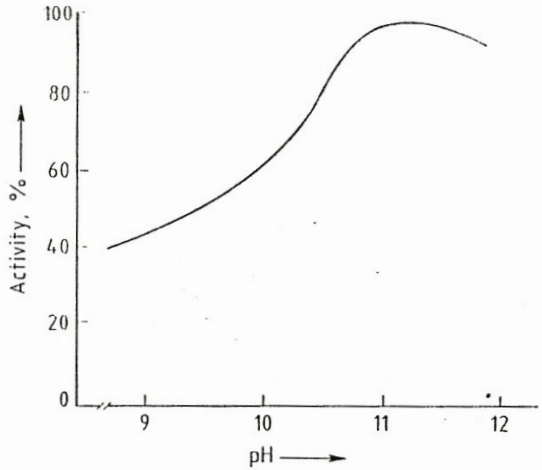


Figure 36. Activity -pH profile of Maxacal, a commercial high-alkaline protease
Activity is determined by incubating the enzyme for 48 min at 40 °C with casein as substrate. The reaction is terminated by adding trichloroacetic acid; the amount of acid-soluble material, measured spectrophotometrically at 260 nm, represents the protease activity (DU).

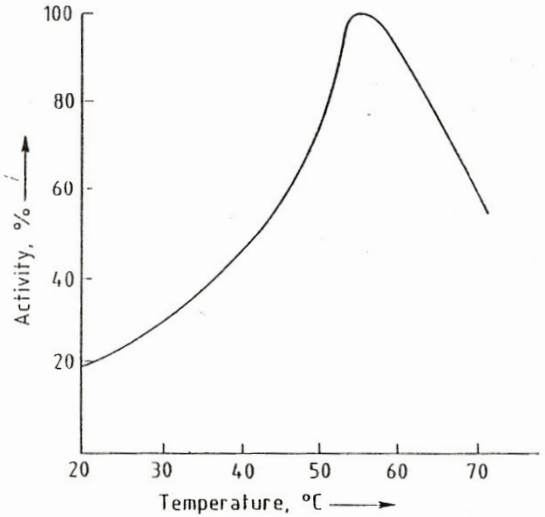
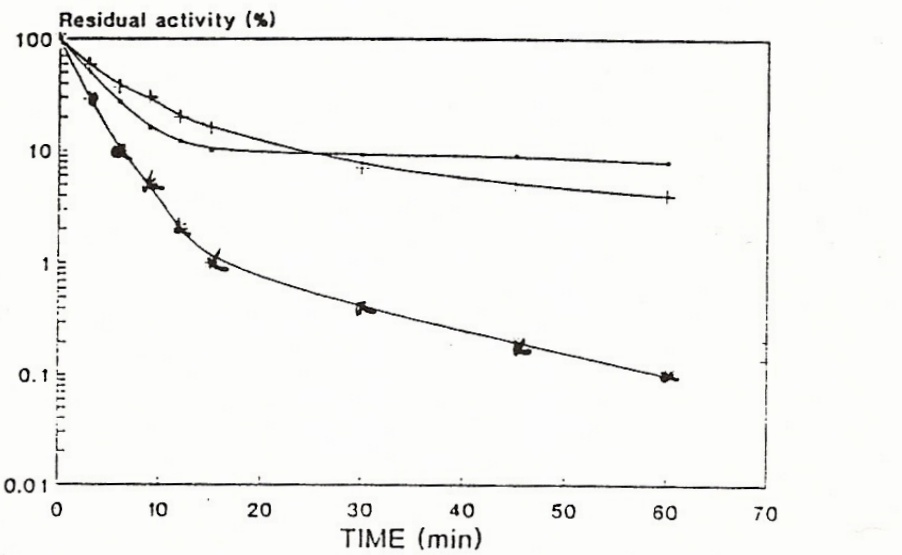


Figure 37. Temperature- activity profile of Maxacal
Activity is determined at pH 10 as described in Figure 36.

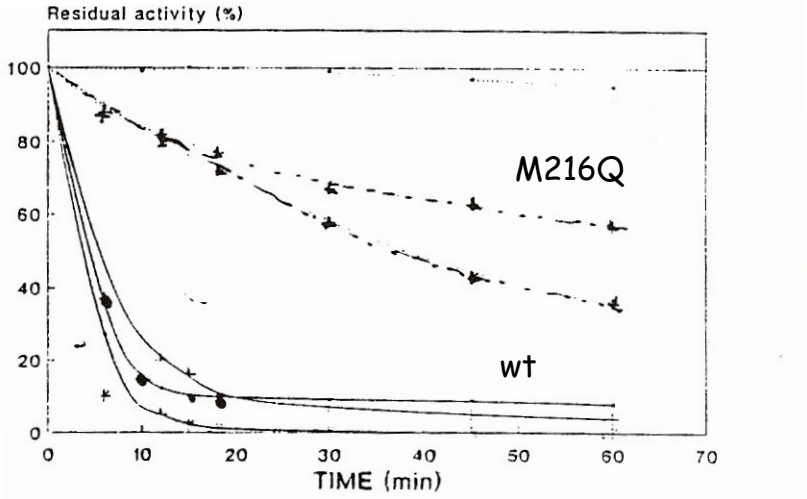
Stabilization of Maxacal to oxidation

Maxacal loses activity in the presence of oxidants such as H_2O_2 or peroxyacids that are commonly found in detergents.
Cysteine and methionine residues are the main targets of oxidants.
Maxacal does not contain cysteines, but it has 3 methionine residues.



1. Residual activity of Maxacal after oxidation with 20 mM H_2O_2 (■), 20 mM $NaBO_3$ + 10 mM Tetra Acetyl Ethylene Diamine (+), and 10 mM diperoxydodecanoic diacid (▲). Activity was measured with succinyl-(L)-Ala-(L)-Ala-(L)-Pro-(L)-Phe-*para*-nitroanilide as a substrate [7].

Stabilization of Maxacal to oxidation. Substitution of methionine 216 makes the protease more resistant to oxidants



Residual activity of Maxacal (solid lines) and [redacted] (dotted lines) after oxidation: (■) 20 mM H_2O_2, (+) 20 mM $NaBO_3$ + 10 mM Tetra Acetyl Ethylene Diamine, (*) 10 mM diperoxydodecanoic diacid (see further legend Figure 4).

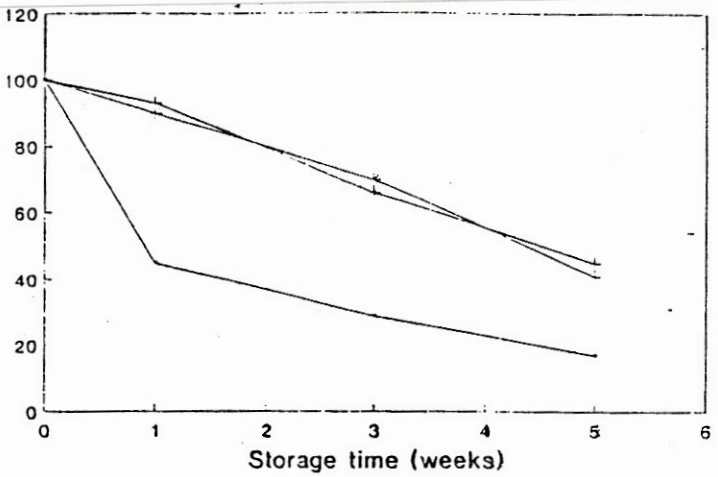


Figure 7. Detergent storage stability of Maxacal (■) and two M216-mutants: M216S (+) and M216Q (*). The storage conditions were 30°C and 80% relative humidity; the detergent contained $NaBO_3$/TAED as a bleaching system. Residual activity was measured with casein as a substrate (see [7] for further experimental details).

In vitro directed evolution to improve the thermostability of a psychrophilic subtilisin

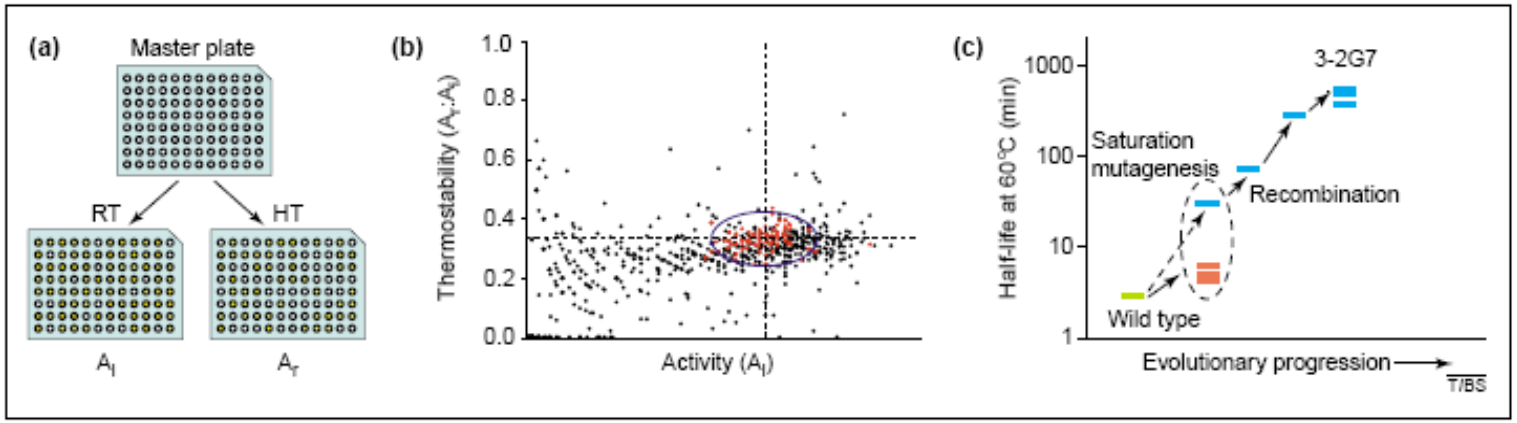


Fig. 3. Directed evolution of a psychrophilic enzyme, subtilisin S41, from the Antarctic bacterium TA41 (Ref. 12). (a) Rapid screening for thermostability and activity is performed by taking replicas from a master plate that contains individual clones. One replica plate is assayed for activity at room temperature (RT), and the second is incubated at high temperature (HT) before activity is measured. The ratio of the residual activity (Ar), calculated from the HT plate, to the initial activity (AI), from the RT plate, provides a measure of thermostability for this irreversibly inactivated enzyme. (b) Activities and stabilities of random S41 mutants[13]. The distribution of wild-type clones measured under the same conditions (red dots inside ellipse) shows the reproducibility of the screen. Mutants distribute well outside this region. Most improvements in activity come at the cost of stability, and vice versa. (c) Progression of the evolution of S41 thermostability, as measured by the half-life of enzyme activity at 60°C, in 1 mM $CaCl_2$. One of the mutants discovered in the first generation contained two amino acid substitutions in a loop region. These were subjected to saturation mutagenesis, and the best mutant was recombined with other random mutants. Further rounds of random mutagenesis and screening produced 3-2G7, which has seven amino acid substitutions.

In vitro directed evolution of the alkaline protease from B. gibsonii to improve activity at low T and thermostability

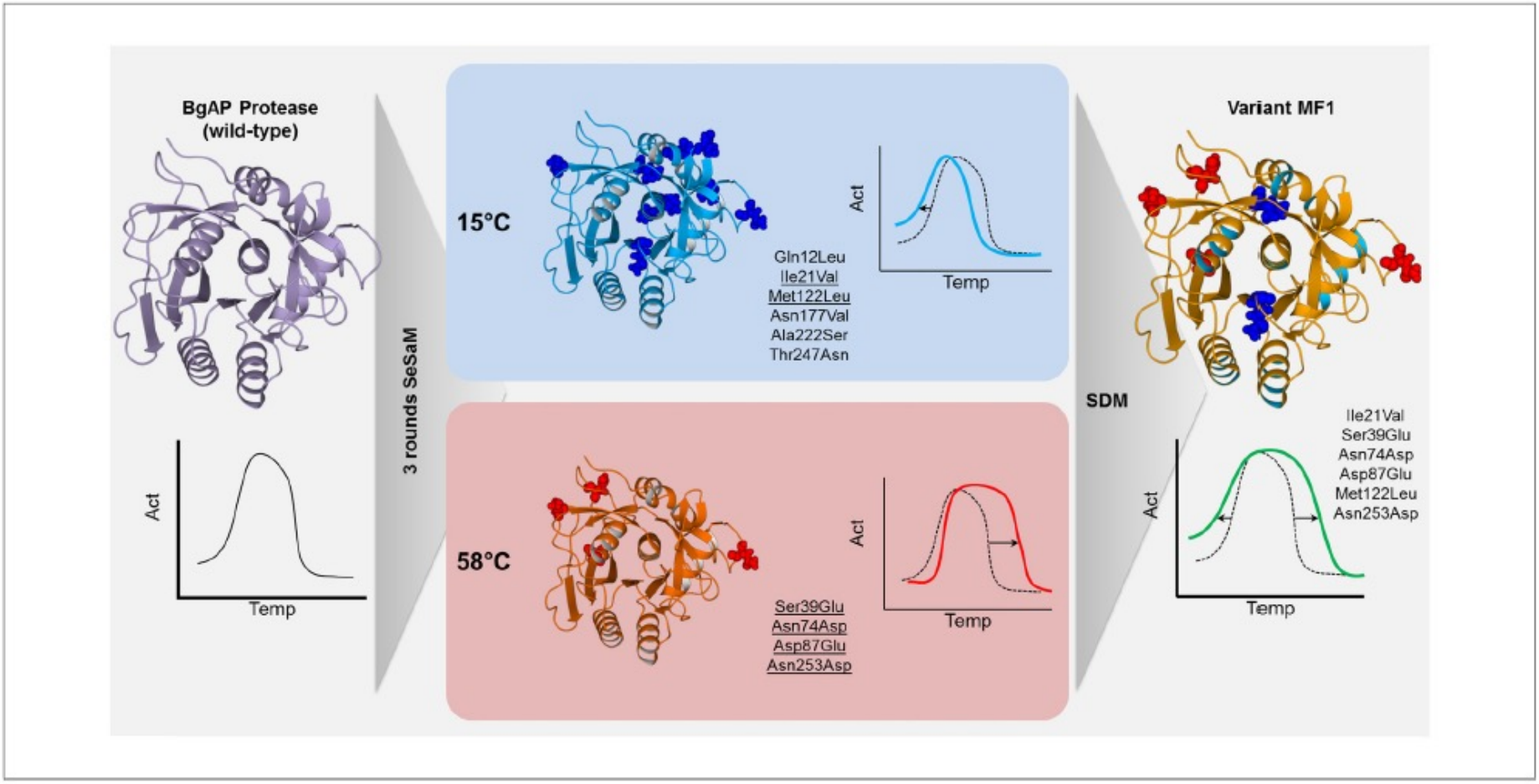


FIGURE 1

Summary of the directed evolution campaign for *Bacillus gibsonii* alkaline protease (BgAP) using sequence saturation mutagenesis (SeSaM) and a parallel screening towards high activity at low temperatures (upper path) and thermal resistance (lower path). Variants with improved temperature-activity profiles were identified, and beneficial amino acid substitutions were combined to generate one single enzyme variant with a wider temperature profile compared to the wild type [9].

Use of proteases in the food industry

- To improve nutritional quality and digestibility of food
- To decrease allergenicity of protein hydrolyzates used as nutritional supplements (es. artificial milk, wheat flour)
- For production of cheese: chymosin and pepsin for casein hydrolysis and rennet formation, mix of proteases for cheese ripening
- To soften meat: papain, bromelain

Food Chemistry 311 (2020) 125911

Contents lists available at ScienceDirect

Food Chemistry

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

A novel catalytic material for hydrolyzing cow's milk allergenic proteins: Papain-$Cu_3(PO_4)_2{\cdot}3H_2O$-magnetic nanoflowers

Nuan Feng[a,b,1], Haiyang Zhang[b,1], Yao Li[c], Yangkaixi Liu[c], Longquan Xu[a], Yi Wang[b], Xu Fei[a,*], Jing Tian[b,*]

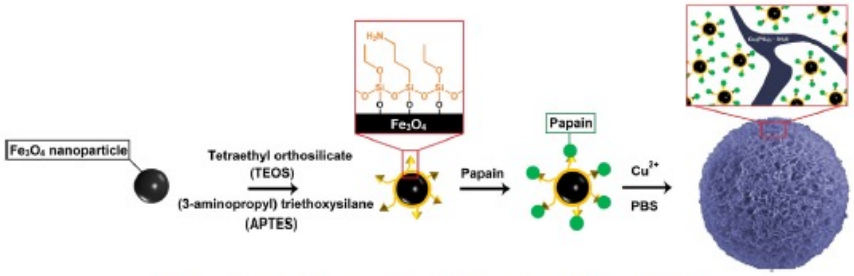


Fig. 1. Schematic diagram of the proposed growth mechanism of PCMN.

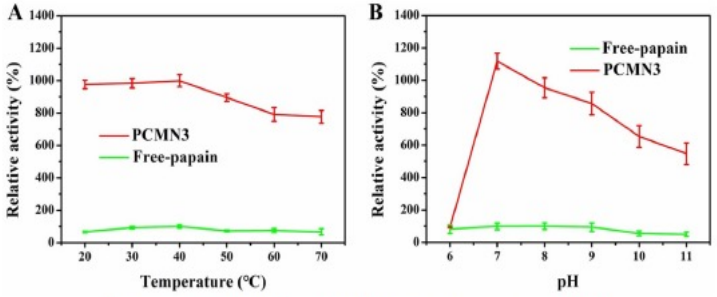
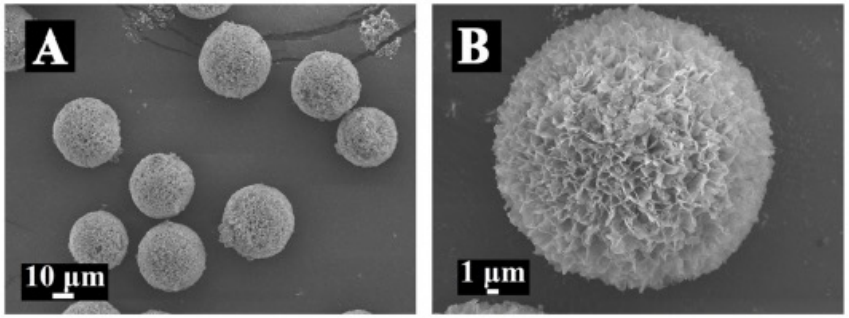


Fig. 4. Thermal stabilities (A) and pH stabilities (B) of free papain and PCMN3.



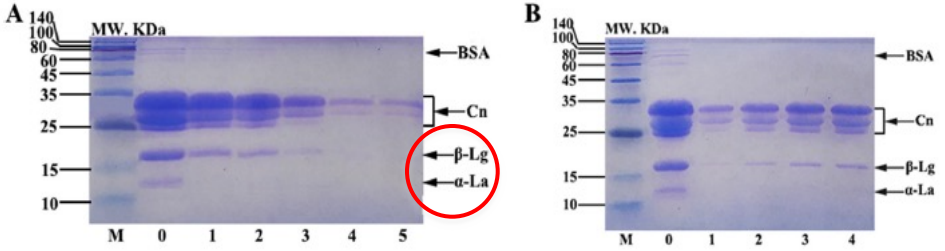


Fig. 5. SDS-PAGE patterns (A) of the hydrolysates, M: protein marker (140–10 KDa); 0:1% (w/w) milk powder aqueous solution; 1–5: the hydrolysates with different reaction times (10 min, 20 min, 30 min, 40 min and 50 min). SDS-PAGE patterns (B) of the hydrolysates from reuse experiment, M: protein marker (140–10 KDa); 0:1% (w/w) milk powder aqueous solution; 1–4: the hydrolysates with different reuse times (first, second, third and fourth time).

Steps in cheese making

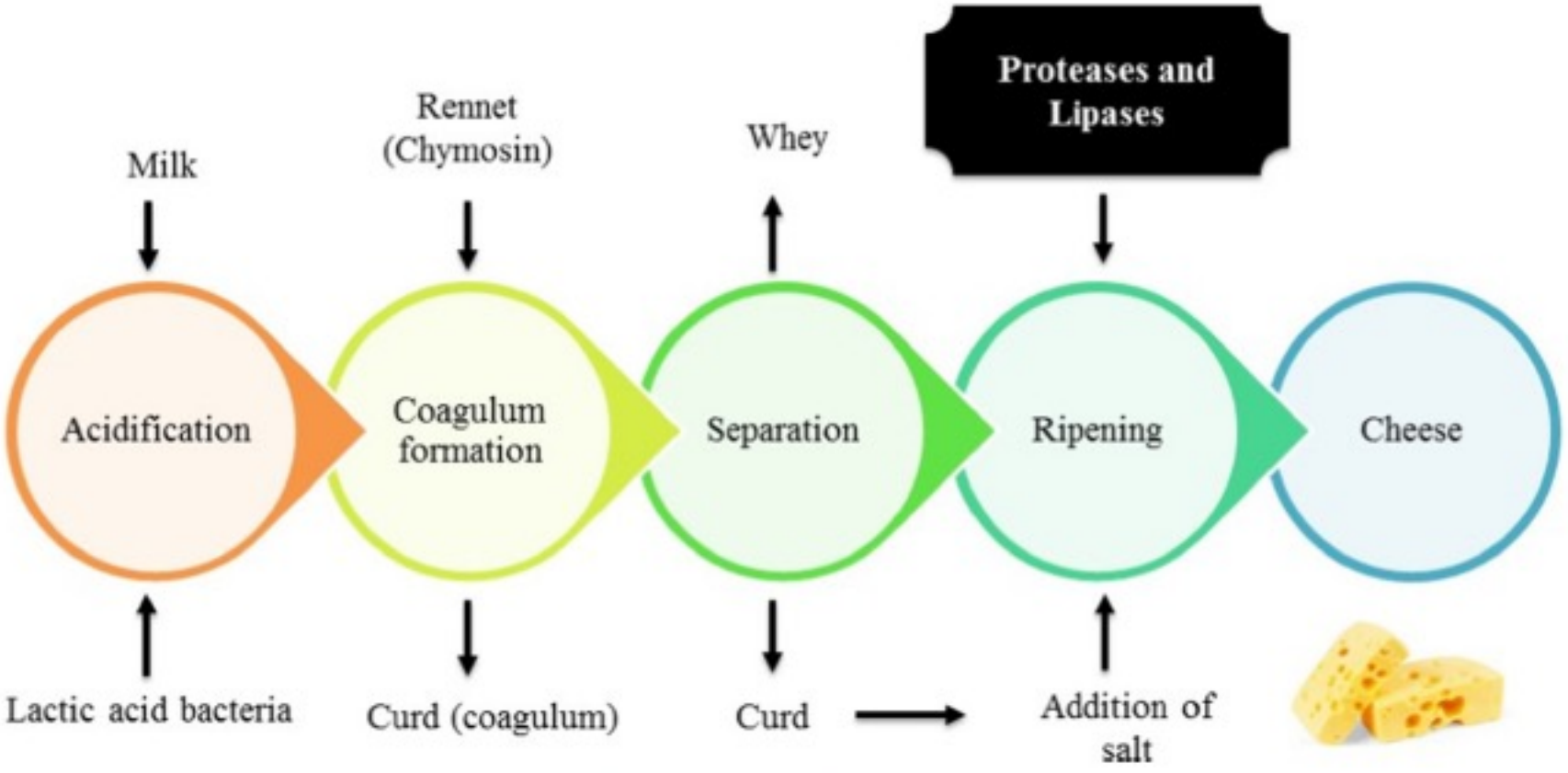
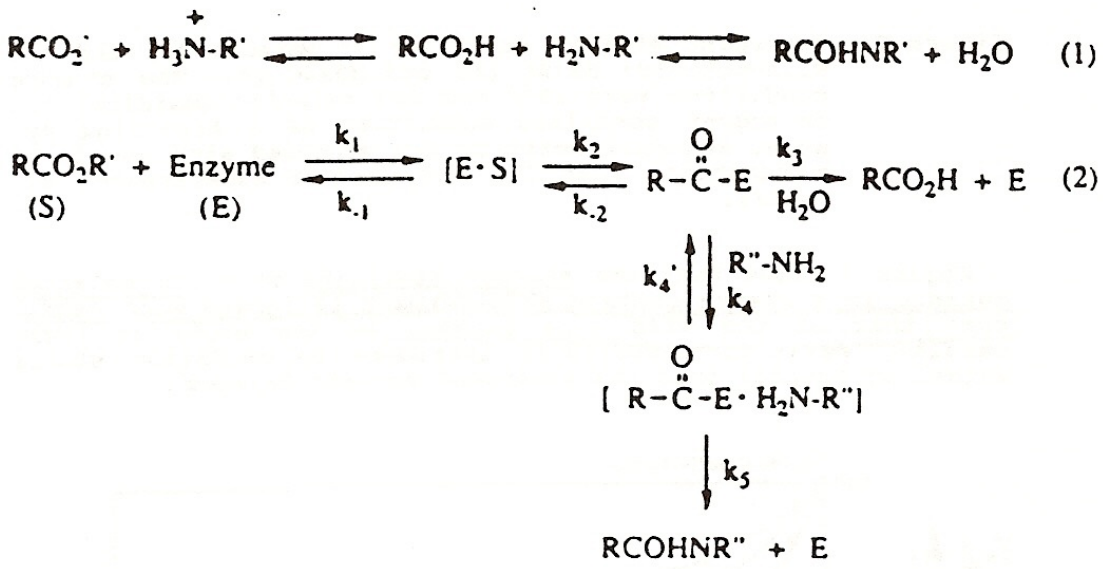


Fig. 6. Steps in cheese making.

Enzymatic synthesis of peptide bonds: transpeptidation

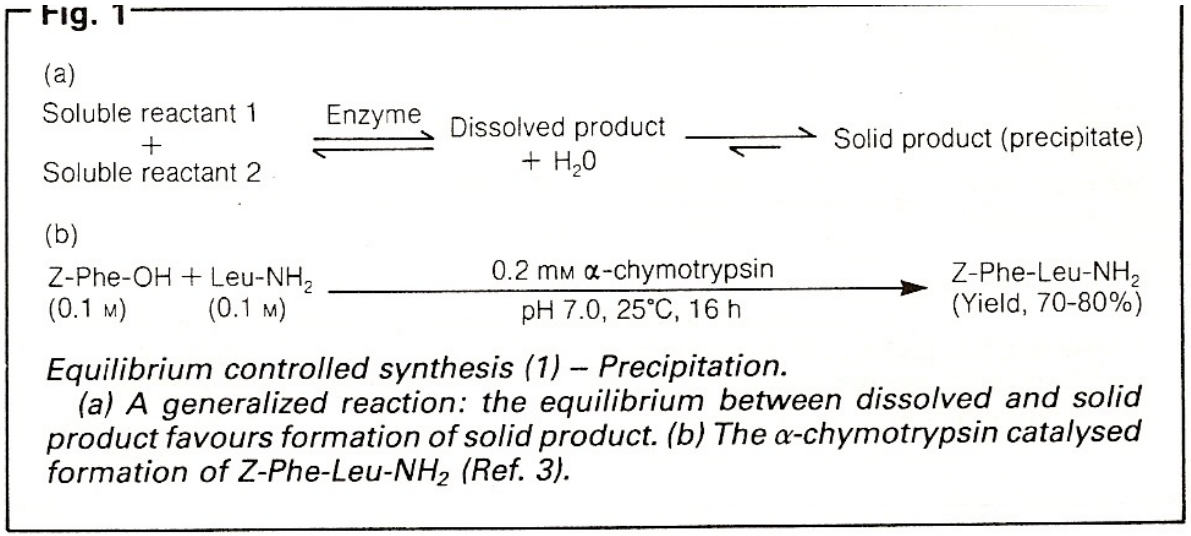
Proteases can catalyze the synthesis of peptide bonds through two approaches:

1. Thermodynamic approach (inverse of the hydrolysis reaction)
2. Kinetic approach (aminolysis reaction)



Strategies for the synthesis of peptide bonds through the thermodynamic approach

The apparent equilibrium is shifted towards synthesis by removal of the peptide product from the reaction mixture: precipitation



Equilibrium controlled synthesis (1) – Precipitation.
(a) A generalized reaction: the equilibrium between dissolved and solid product favours formation of solid product. (b) The α-chymotrypsin catalysed formation of Z-Phe-Leu-NH_2 (Ref. 3).

Strategies for the synthesis of peptide bonds by proteases

Chemical groups that block –NH2 and -COOH are used to confer directionality to the bond and to modify solubility of reagents and products

NH_2 group
Z group: benzyloxycarbonyl
Ac group: acetyl

COOH group
Obu^t group: tert-butyl ester
NH_2 group: amide

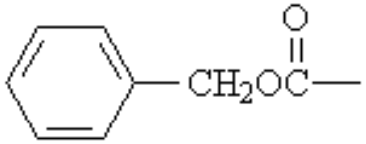


Table 2
Effect of blocking residues on product solubility

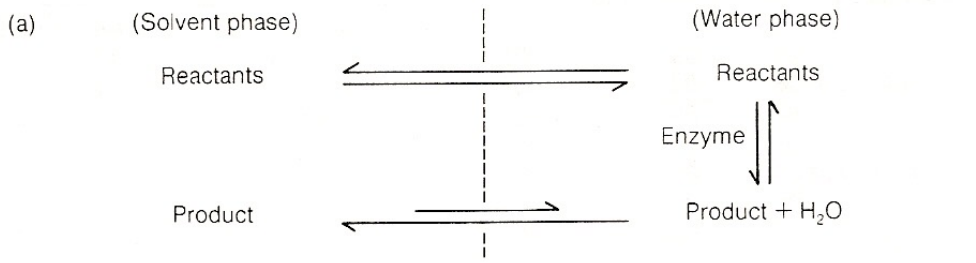
Product	Solubility[a]/ μM
Ac-Phe-Leu-NH_2	10^4 (in water)
Z-Phe-Leu-NH_2	27
Z-Phe-Leu-OEt	17
Z-Phe-Leu-OBu^t	1.7
Z-Phe-Leu-NHC_6H_5	0.8
Z-Phe-Leu-ODPM	0.03

[a]In pH 7.0 buffer containing 10% dimethylformamide.

Strategies for the synthesis of peptide bonds through the thermodynamic approach

The apparent equilibrium is shifted towards synthesis by removal of the peptide product from the reaction mixture: Biphasic system: water + immiscible solvent

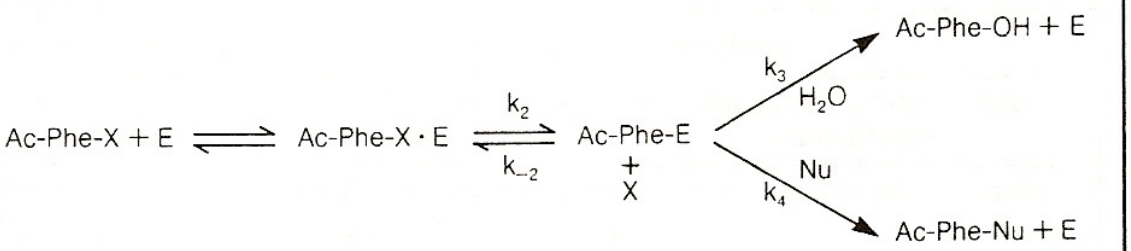
Fig. 2



Equilibrium controlled synthesis (2) – Biphasic system.
(a) A generalized reaction: the low concentration of product in the aqueous phase means that equilibrium in the aqueous phase is not reached until there are high concentrations of product in the solvent. (b) The α-chymotrypsin catalysed synthesis of Ac-Trp-Leu-NH_2 (Ref. 1).

Strategies for the synthesis of peptide bonds through the kinetic approach

Fig. 4



Kinetically controlled synthesis.
The catalytic mechanism of α-chymotrypsin: the covalent-linked enzyme-peptide intermediate (Ac-Phe-E) is subjected to nucleophilic attack from H_2O or other nucleophiles (Nu): the rates of the reactions (k_3 and k_4) dictate its outcome.

This approach can be used with serine and cysteine proteases that form a covalent acyl-enzyme intermediate.
Esters or amides of amino acids are used as acyl group donors to form the acyl-enzyme.
Deacylation takes place as competition between hydrolysis and aminolysis.
The aminolysis reaction is favoured in organic solvents.

Enzymes are able to retain catalytic activity in organic solvents

Enzymes retain catalytic activity in organic solvents because they retain a thin shell of water molecules.

Enzymes maintain the pre-existing ionization state before transfer in the organic solvent

→ pH memory.

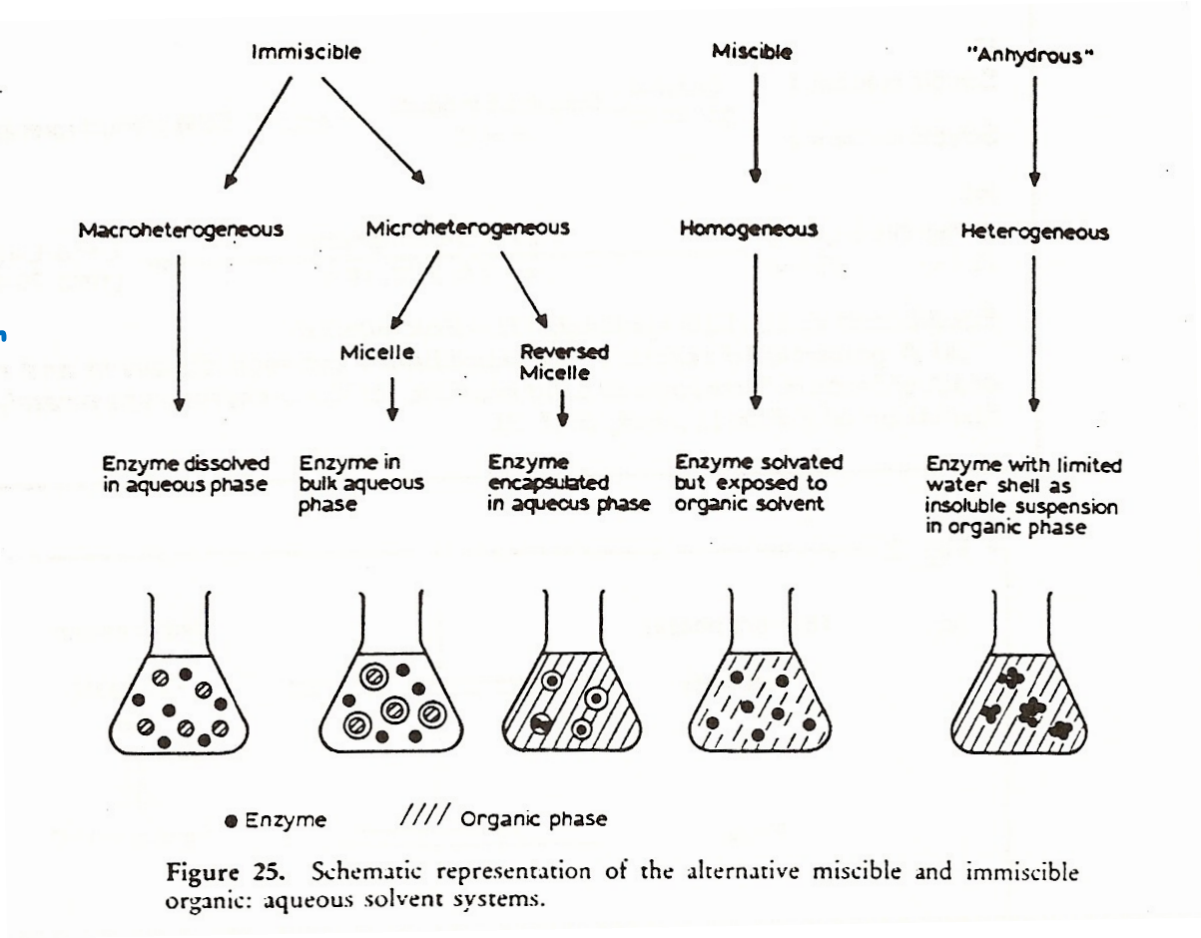


Figure 25. Schematic representation of the alternative miscible and immiscible organic: aqueous solvent systems.

Solvent-mediated inversion of the enantioselectivity of an enzyme

Transesterification reaction catalyzed by *Aspergillus oryzae* protease in different organic solvents
N-acetyl- (D or L)-phenylalanine-chloroethylester + propanol

Solvente	Enantioselettività (V_L/V_D)
Acetonitrile	7.1
Dimetilformamide	5.7
Acetone	1.3
Toluene	0.26
Ottano	0.24

Binding mode of D substrate is productive in organic solvent because the hydrophobic Phe side chain is exposed

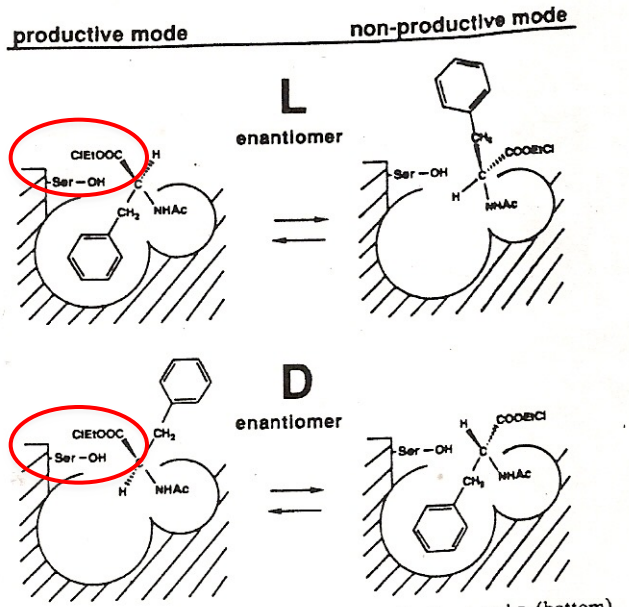


Figure 1. Schematic representation of binding of L (top) and D (bottom) enantiomers of **1** to the active center of *A. oryzae* protease. The bigger unfinished circle depicts the hydrophobic binding pocket. The serine residue shown is the head nucleophile of the enzyme;[4] its hydroxyl is properly aligned to attack the ester carbonyl only in the productive binding mode.

Strategies for the enzymatic synthesis of peptides

Table 5
Strategy for peptide synthesis by enzymatic method

	Equilibrium controlled synthesis	Kinetically controlled synthesis
Type of enzymes	All proteases (proteinases)	Serine and thiol proteases
Size of peptides (amino acid residues)	2–10 (Precipitation) 2–10 (Biphasic) 2–100 (Dissolved)	2–100
Specificity requirement in fragment (> hexapeptides) condensation	Strict	Not strict

Experimental conditions for enzymatic peptide bond synthesis

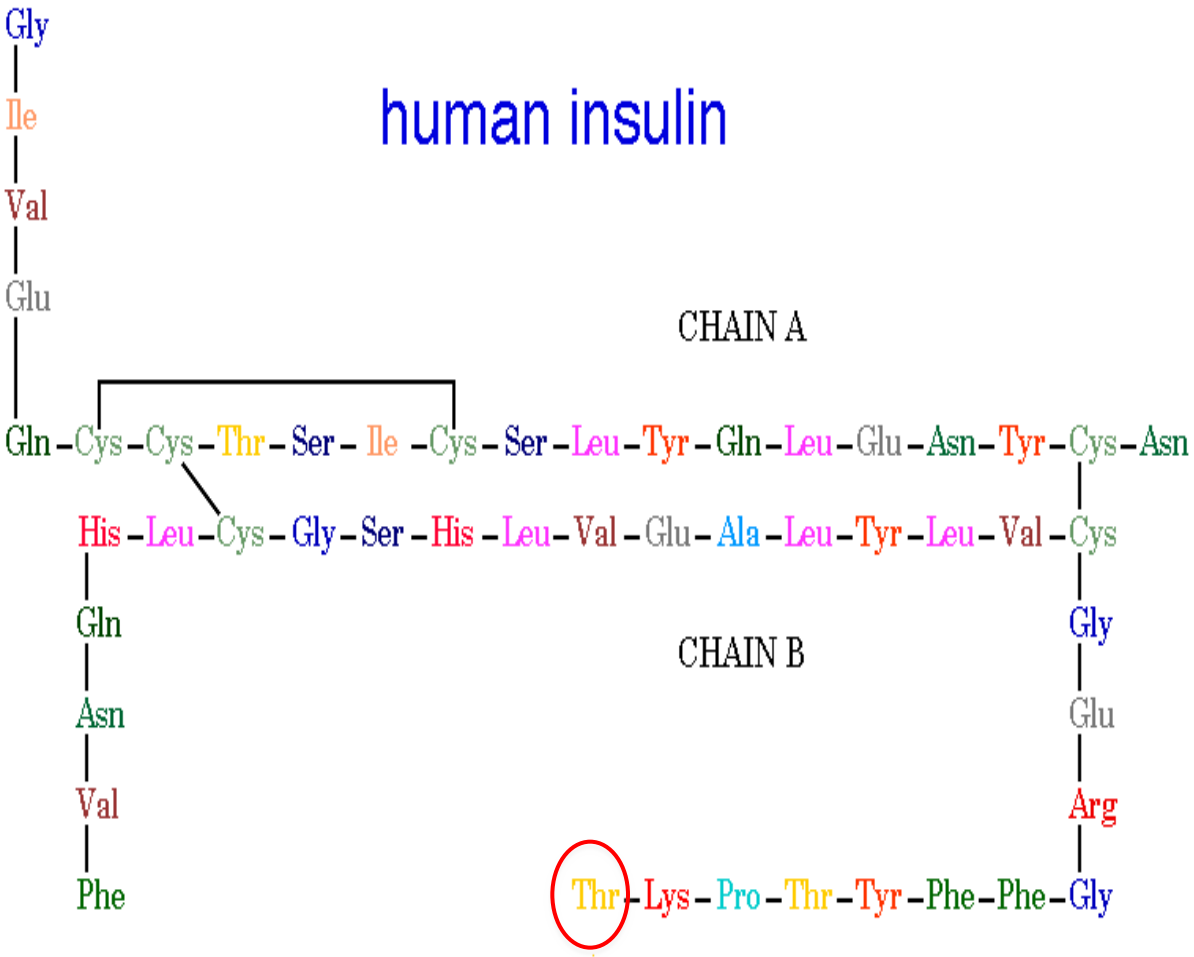
Table 3
Experimental conditions for peptide bond synthesis[a]

Enzyme	Carboxyl component (Concn., M)	Amine component (Concn., M)	pH	Enzyme conc. (μM)	Reaction time (h)	Yield (%)
		Equilibrium controlled processes				
α-Chymotrypsin	Z-Phe-OH (0.05)	Leu-NH_2 (0.05)	7	200	20	72
Trypsin	Bz-Arg-OH (0.05)	Leu-OBu^t (1.0)	6.5	100	20	60 (soluble)
Subtilisin	Z-Gly-Pro-Leu-OH (0.05)	Leu-NHC_6H_5 (0.05)	7	100	20	54
Papain[b]	Z-Ala-OH (0.05)	Leu-ODPM (0.05)	5	10	5	75
Papain	Z-Arg-OH (0.05)	Leu-OBu^t (1.0)	5.5	8	5	50 (soluble)
Thermolysin	Z-Phe-OH (0.05)	Leu-NH_2 (0.05)	7	3	5	70
Pepsin	Z-Gly-Phe-OH (0.05)	Leu-NHC_6H_5 (0.05)	4.5	200	20	70
		Kinetically controlled processes				
α-Chymotrypsin	Ac-Phe-OEt (0.1)	Leu-NH_2 (0.1)	10	10	0.03 (2 min)	80
Trypsin[c]	Bz-Arg-OEt (0.2)	Leu-NH_2 (0.2)	10	10	0.03 (2 min)	70 (soluble)
Carboxypeptidase Y[d]	Bz-Ala-OMe (0.055)	Val-NH_2 (0.6)	9.7	4.5	1	80
Papain	Bz-Ala-OMe (0.1)	Val-NH_2 (0.2)	8	200[e]	1	82

[a]Data from Ref. 3. Except where otherwise specified, the reaction was carried out at 37 or 25°C using commercially available crystalline enzyme. The reaction with papain was performed in the presence of 10–50 mM KCN and 5 mM EDTA or 0.2 mM EDTA and 0.2 M dithiothreitol (for kinetically controlled process).
[b]The reaction mixture contained 40% dimethylformamide.
[c]The reaction mixture contained 20% dimethylformamide.
[d]Highly purified preparation.
[e]Crude preparation from Merck, 3.5 U/mg.

Semi-synthesis of human insulin from porcine insulin

Human insulin differs from porcine insulin only for the last residue of the B chain:
Thr B30 vs Ala B30.

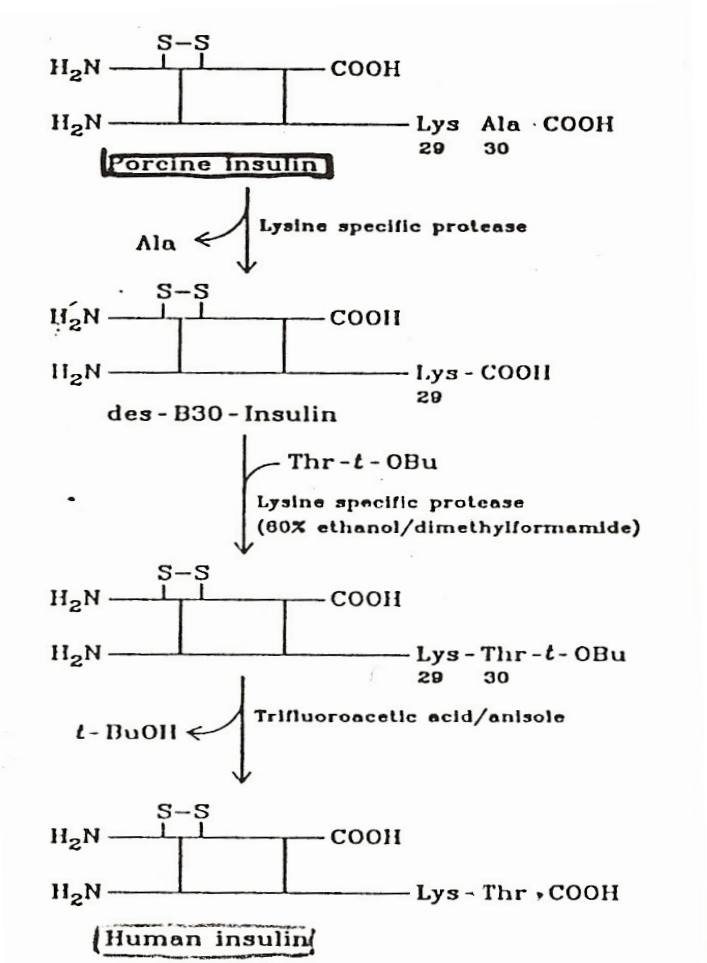


Semi-synthesis of human insulin from porcine insulin

Ala30 is removed by carboxypeptidase or by a lysine-specific protease.
A lysine-specific protease is used to form the Lys29-Thr peptide bond.

Procedure patented by Novo in 1982.

Transpeptidation of recombinant insulin (B chain lacking Thr30) produced in S. cerevisiae.

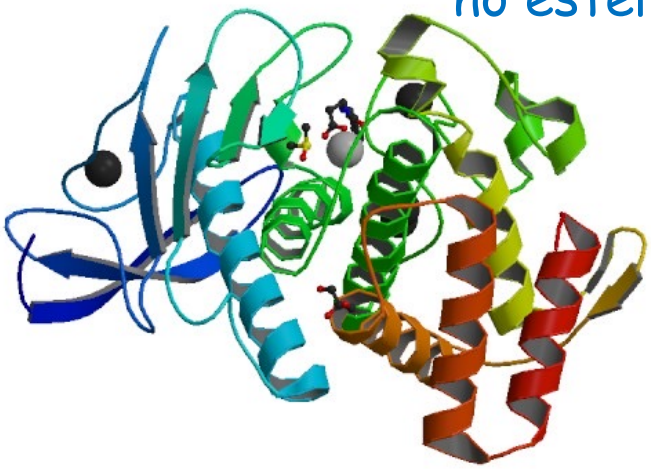


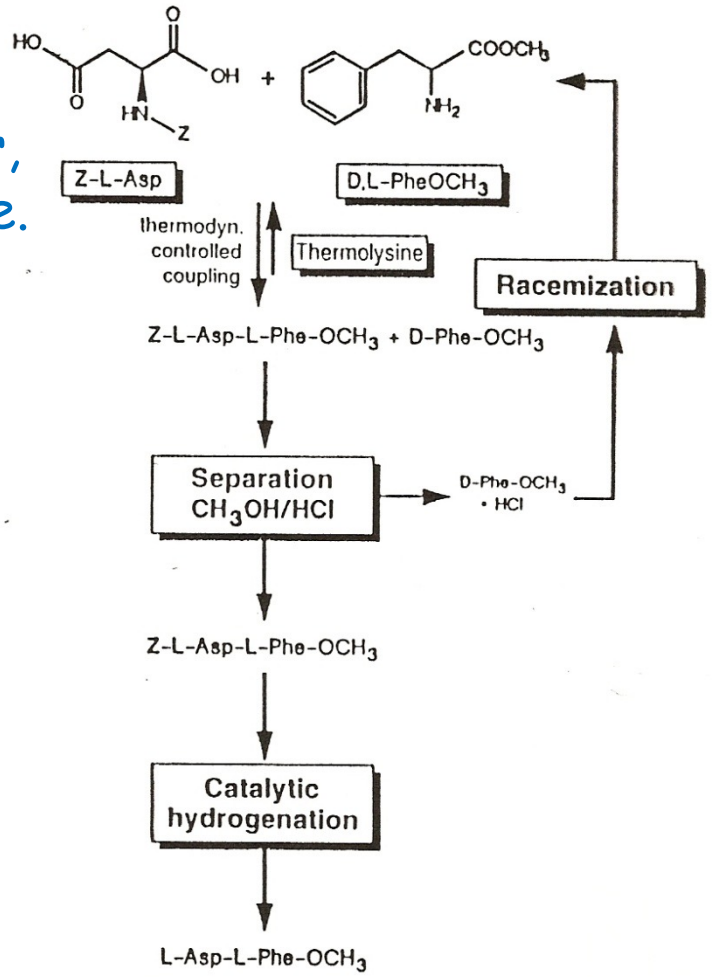
. Enzymatic preparation of human insulin from porcine insulin.

Synthesis of aspartame

Aspartame is a dipeptide formed by aspartate and phenylalanine-methylester, it is 100-200 times sweeter than sucrose. Peptide bond synthesis is catalyzed by the metalloprotease thermolysin.

Thermolysin: enantiospecific (D,L-Phe)
regiospecific (α isomer)
thermostable (50-60° C)
no esterase activity





Tosoh process for the enzymatic manufacture of aspartame (OYAMA and KIHARA, 1984).