Achievement of Protein Thermostability by Amino Acid Substitution

2018/6/30 M1 Majima Sohei

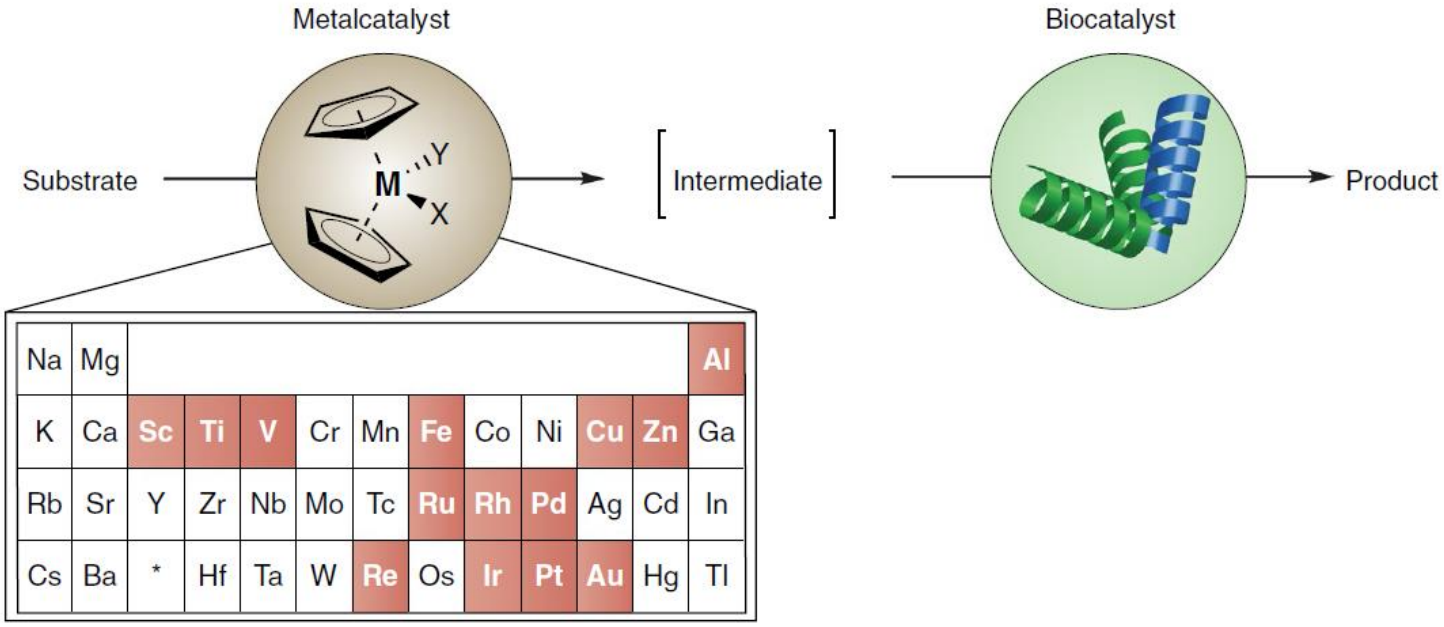
Contents of Today's seminar

- Introduction
- Study on hyperthermophilic enzymes to understand the origin of thermostability
- Acquiring thermostability by amino acid substitution
- Summary

Introduction

New type of synthesis:

combination of chemocatalyst and biocatalyst

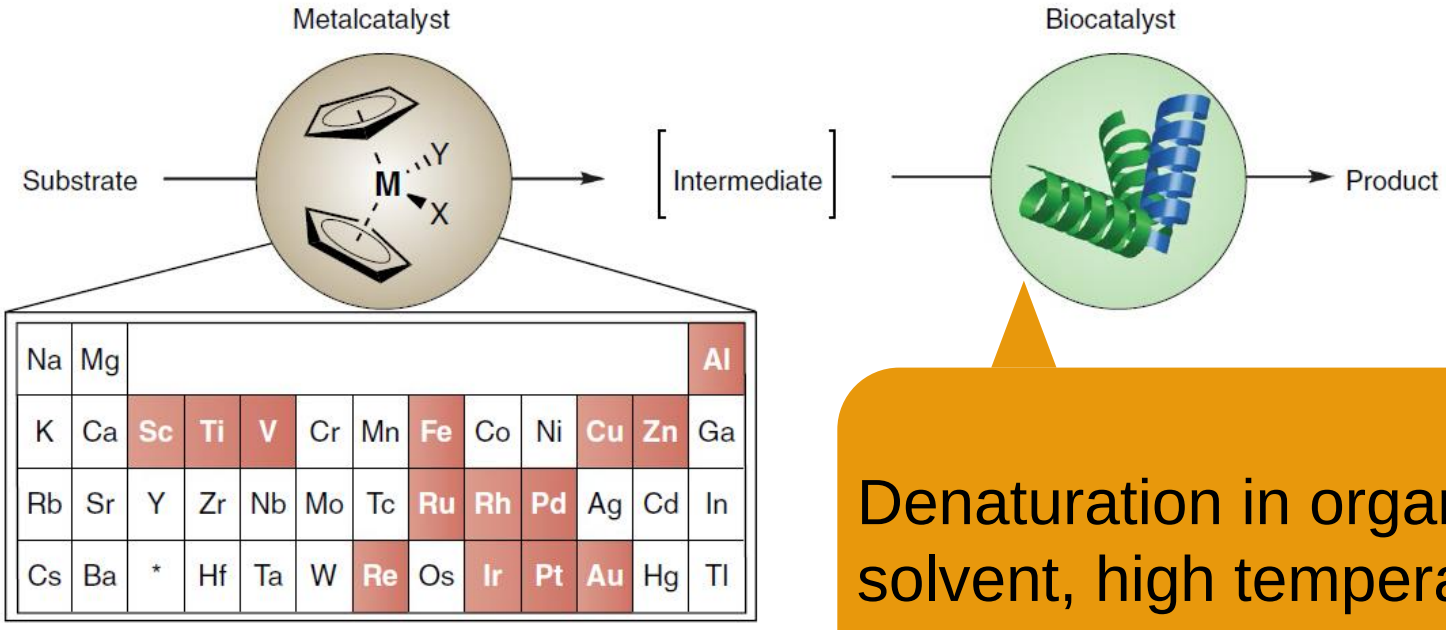


Bornscheuer *et al*. Nature Catalysis, 2018, *1*, 12

Introduction

New type of synthesis:

combination of chemocatalyst and biocatalyst

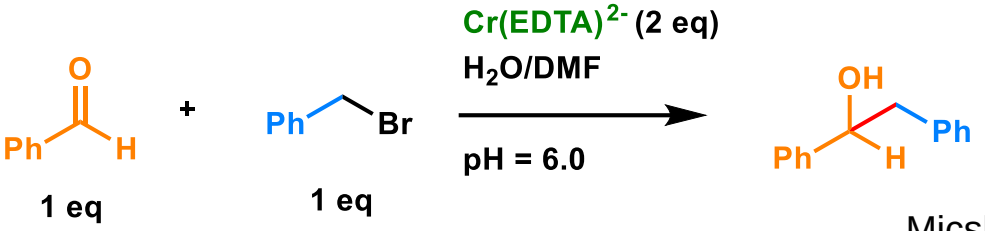


Denaturation in organic solvent, high temperature

Bornscheuer *et al*. Nature Catalysis, 2018, *1*, 12

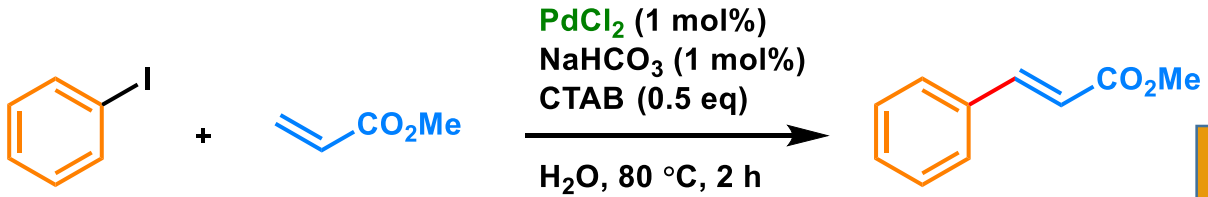
Introduction

Organic reactions in aqueous media

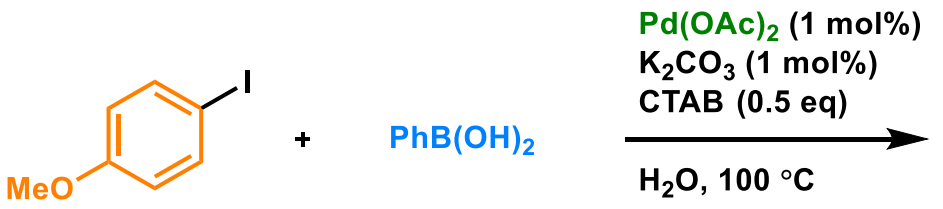


NHK reaction

Micskei *et al*. Tetrahedron Lett. 2001, *42*, 7711



Heck reaction



Suzuki-Miyaura coupling

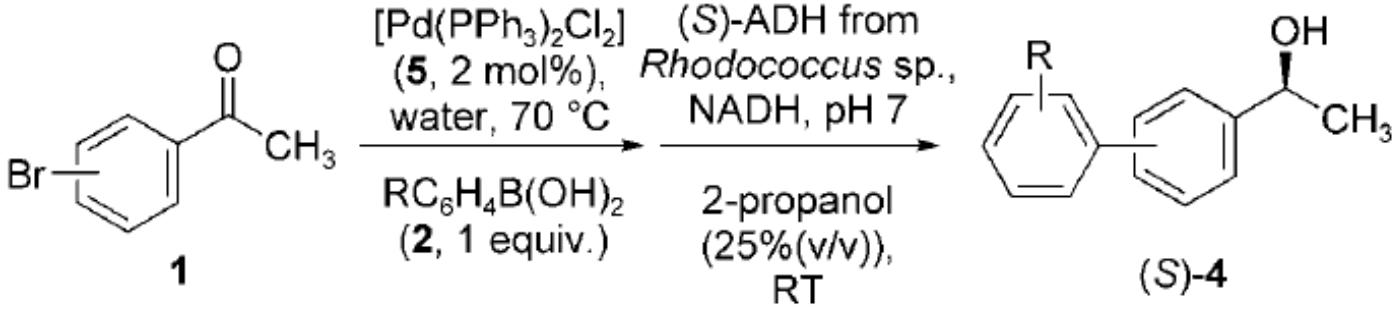
*CTAB = $C_{16}H_{32}NMe_3Br$, amphiphilic molecule

Bhattacharya *et al*. Tetrahedron Lett. 2005, *46*, 3557

Introduction

Examples of the reactions

Table 1: Substrate spectrum of the one-pot two-step synthesis.[a]



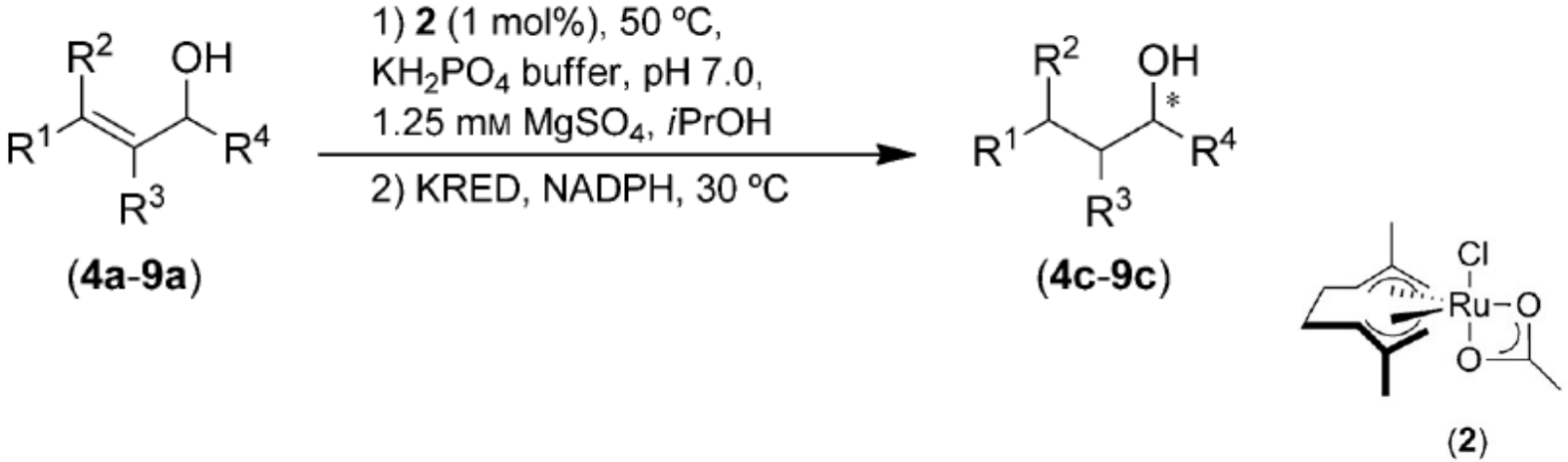
combination of Suzuki-Miyaura coupling and enzymatic stereoselective reduction of ketone

✔Cool down, pH adjustment is necessary

Burda *et al*, Angew. Chem. Int. Ed. 2008, *47*, 9551

Introduction

Examples of the reactions



combination of Ru-catalyzed olefin isomerization and enzymatic stereoselective reduction of ketone.

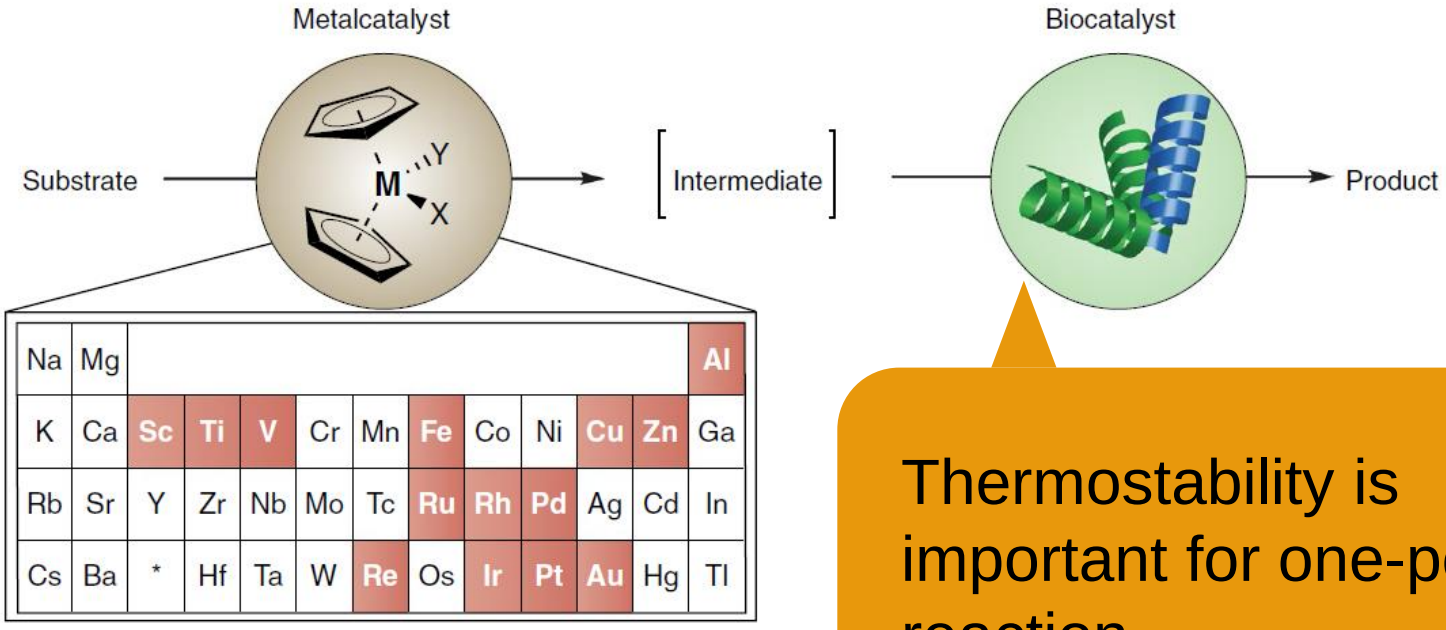
✔Cool down is necessary

Sabin, *et al*. Angew. Chem. Int. Ed. 2016, *55*, 8691

Introduction

New type of synthesis:

combination of chemocatalyst and biocatalyst



Thermostability is important for one-pot reaction

Bornscheuer *et al*. Nature Catalysis, 2018, *1*, 12

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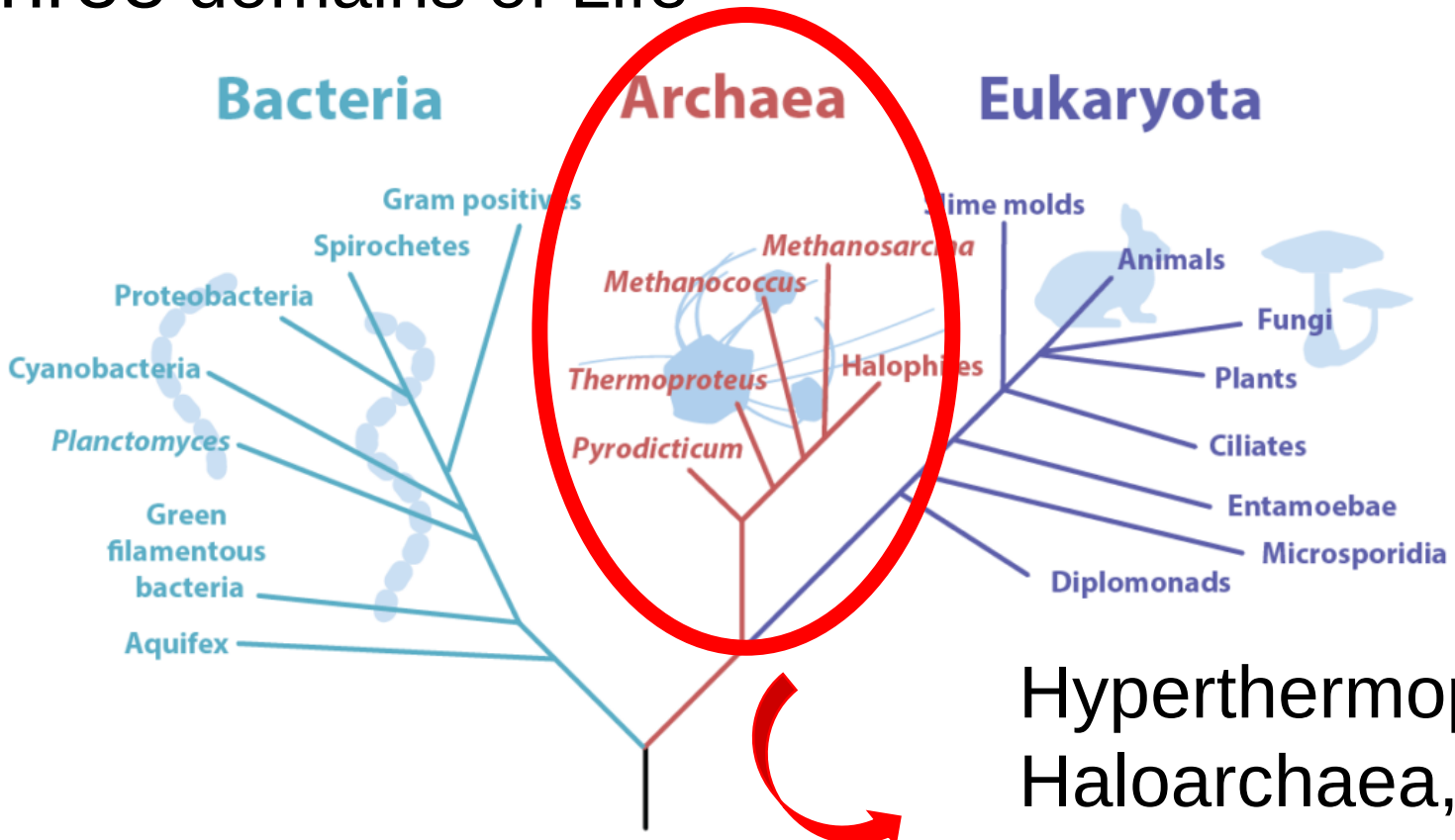
What are Hyperthermophiles?

Three domains of Life



Hyperthermophiles,
Haloarchaea,
Methanogen …

https://openoregonstate.pressbooks.pub/microbiology/chapter/archaea/

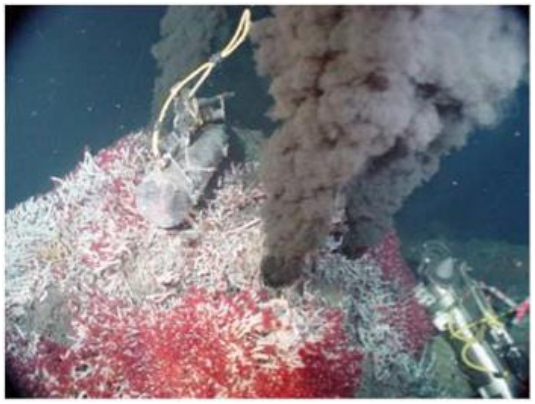
Woese *et al*, Proc. Natl. Acad. Sci. USA 87 (1990)

What are Hyperthemophiles?

Hyperthermophiles live under extremely hot conditions (>80 ℃)

TABLE 1. Hyperthermophile diversity

Organism (references)	Growth conditions	Isolation/habitat	Metabolic properties
Acidianus infernus (301)	90°C, pH 2.0, 0.2% NaCl	Hot water, mud, and marine sediments at hot springs in Italy, the Azores, and the United States	Facultative aerobe, obligate chemolithotrophic growth by S^0 oxidation (aerobic) or by S^0 reduction with H_2 (anaerobic)
A. ambivalens (106, 384)	80°C, pH 2.5	Solfataric source, Leirhnukur fissure, Iceland	Facultative anaerobe, chemolithoautotroph; uses either $S^0 + O_2$ (yielding H_2SO_4) or $S^0 + H_2$ (yielding H_2S) as energy source.
Thermoproteales			
Thermoproteus tenax (33, 382)	88°C, pH 5.0	Solfataric fields, Iceland	Anaerobe, facultative chemolithoautotroph; heterotrophic growth on glucose, starch, glycogen, a few alcohols, a few organic acids, peptides, and formamide by S^0 respiration; H_2S required; produces acetate, isovalerate, and isobutyrate from peptone + S^0
T. neutrophilus (104, 295)	85°C, pH 6.8	Hot spring, Iceland	Anaerobe, facultative autotroph; acetate >> succinate > propionate can be used as carbon sources
T. uzoniensis (33)	90°C, pH 5.6	Uzon caldera, Kamchatka peninsula	Anaerobe; ferments peptides, producing acetate, isovalerate, and isobutyrate; S^0 stimulates growth.
Pyrobaculum islandicum (148)	100°C, pH 6.0	Geothermal power plant, Iceland	Anaerobe, facultative heterotroph (growth on peptide substrates with S^0, $S_2O_3^{2-}$ sulfite, L-cystine, or oxidized glutathione as electron acceptors; grows chemolithoautotrophically on CO_2, $S^0 + H_2$, (produces H_2S)
P. organotrophum (148)	102°C, pH 6.0	Solfataric fields, Iceland, Italy, and Azores	Anaerobe, obligate heterotroph; growth on peptide substrates with S^0, L-cystine, or oxidized glutathione as electron acceptor
P. aerophilum[b] (355)	100°C, pH 7.0, 1.5% NaCl	Shallow marine boiling-water holes, Iischia, Italy	Grows by aerobic respiration or by dissimilatory nitrate reduction; heterotrophic growth on peptide substrates, propionate, and acetate; autotrophic growth by H_2 or $S_2O_3^{2-}$ oxidation; S^0 inhibits growth

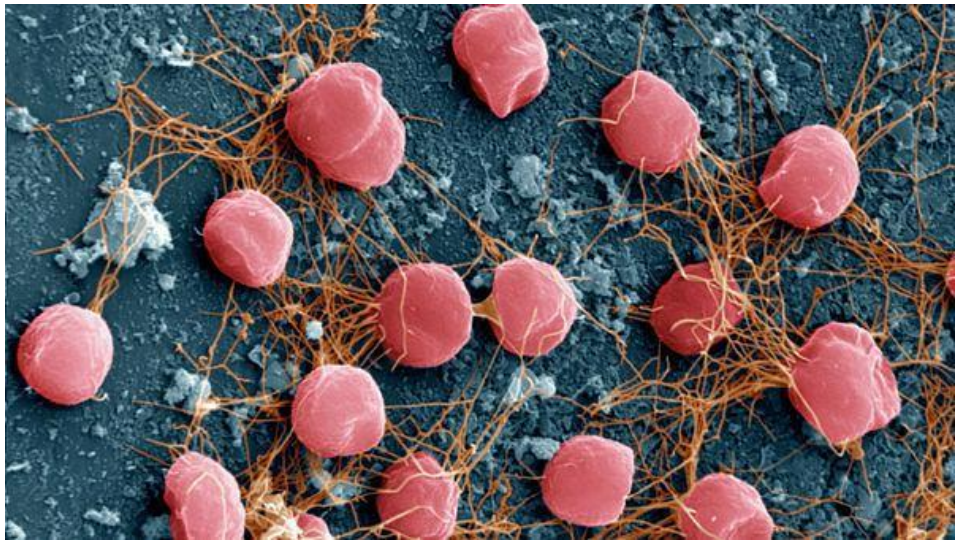


Hydrothermal vent in deep sea.

https://www.brh.co.jp/communication/shinka/2015/post_000022.html

Zeikus *et al*. MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS. 2001, 65, 1

Application of Hyperthemophiles



The DNA polymerase of this archaea is used for PCR.

Pyrococcus furiosus
Optimum growth temperature: 100 ℃

Why are these proteins stable to heat ???

https://alchetron.com/Pyrococcus-furiosus

https://rechtsmedizin.med.uni-rostock.de/arbeitsbereiche/forensische-genetik/

The origin of protein stability

thermophilic

mesophilic

Comparison of homolog proteins

Stability comes from several factors such as;

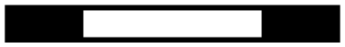
- Hydrophobic interactions
- Solvent Accessible Surface Area
- Ion pairs
- Hydrogen bond
- Cation-π interaction
- Covalent bond and so on …

Hydrophobic interactions

One of the most important factor

Study on Adenylate Kinase(AK)

AK_{the} , AK_{jan} : thermophilic AK_{vol}: mesophilic

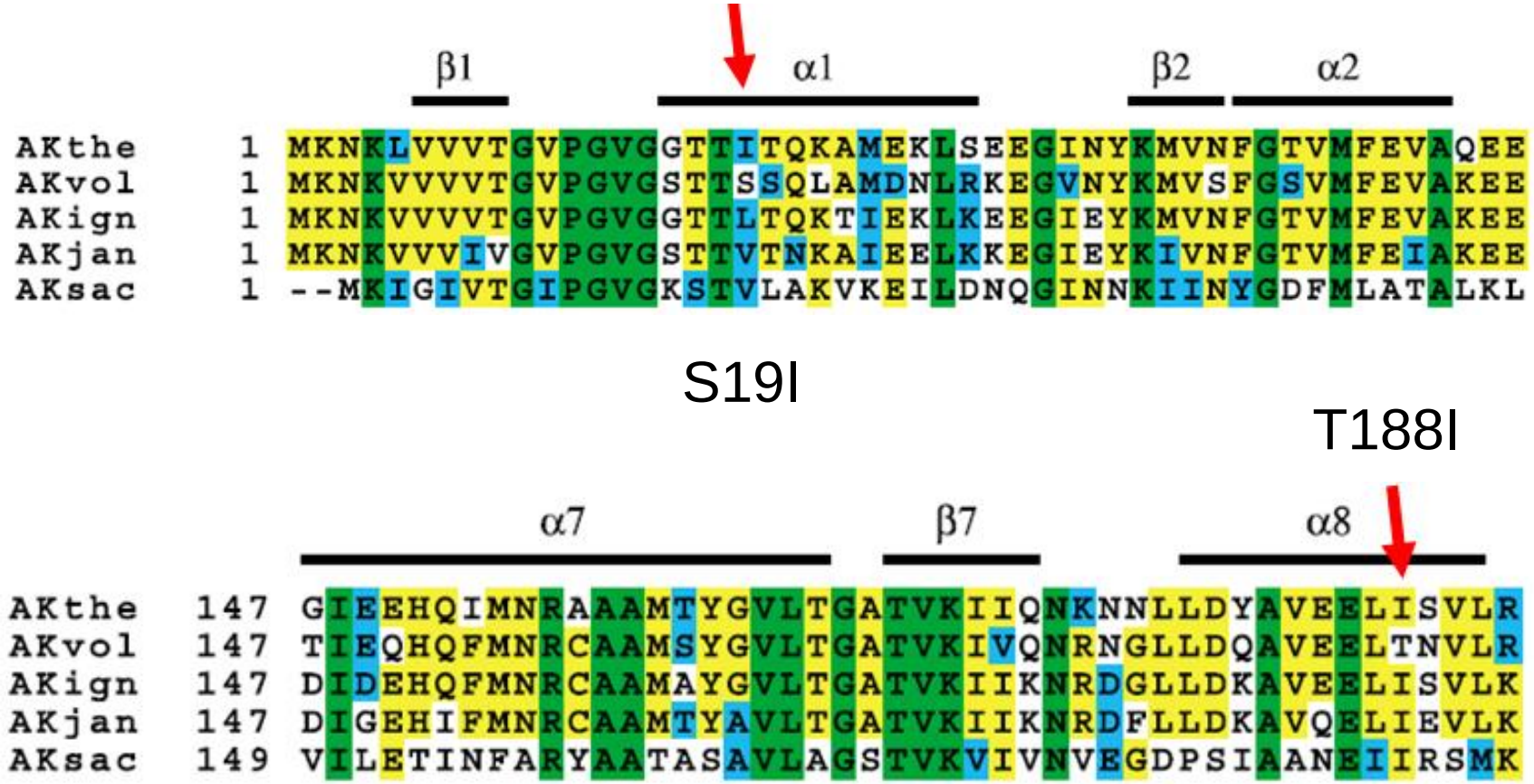
	Construction	Temperature optimum	T_m	ΔT_m
AKvol		37 °C	69 °C	0 °C
J36V		45 °C	73 °C	+4 °C
V160J		53 °C	74 °C	+5 °C
JVJ		60 °C	89 °C	+20 / 0 °C
AKjan		83 °C	103 °C	0 °C
AKthe		68 °C	86 °C	

amino acid sequence

Philips *et al*, J. Mol. Biol. 2003, *330*, 1087

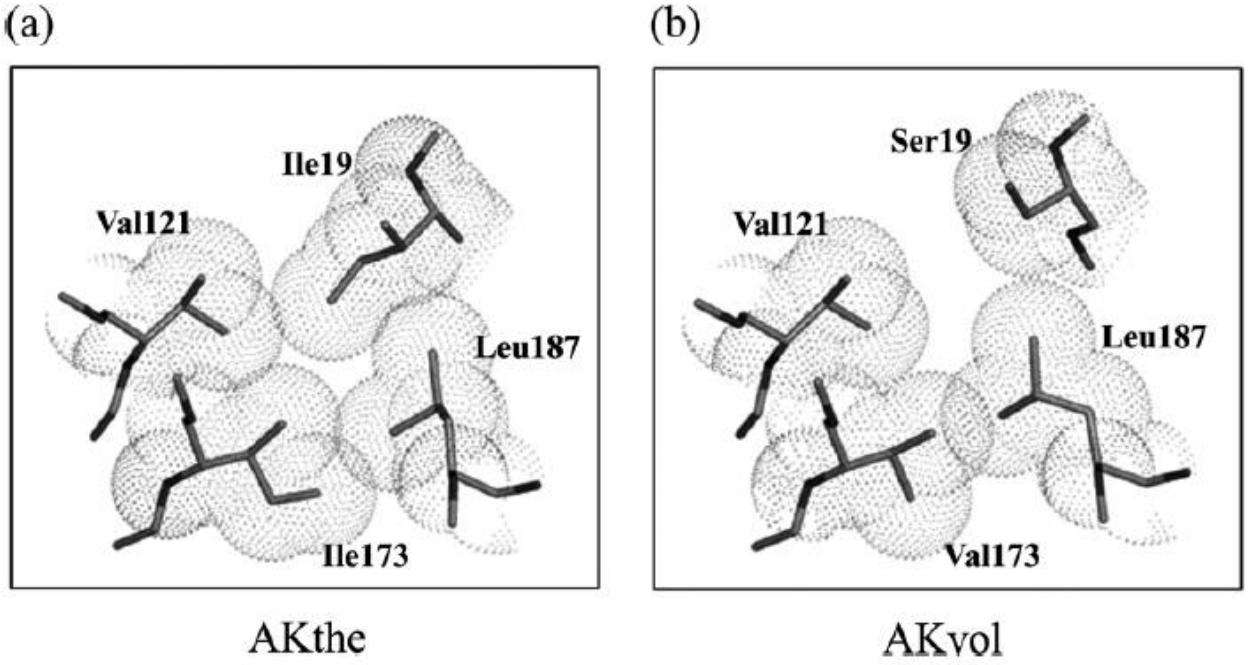
Hydrophobic interactions

Comparison of sequence



Philips *et al*, J. Mol. Biol. 2003, *330*, 1087

Hydrophobic interactions



N-terminal and C-terminal is stabilized by hydrophobic interaction.

Philips *et al*, J. Mol. Biol. 2003, *330*, 1087

Solvent Accessible Surface Area

Study on O^6-methylguanidine-DNA methyltransferase

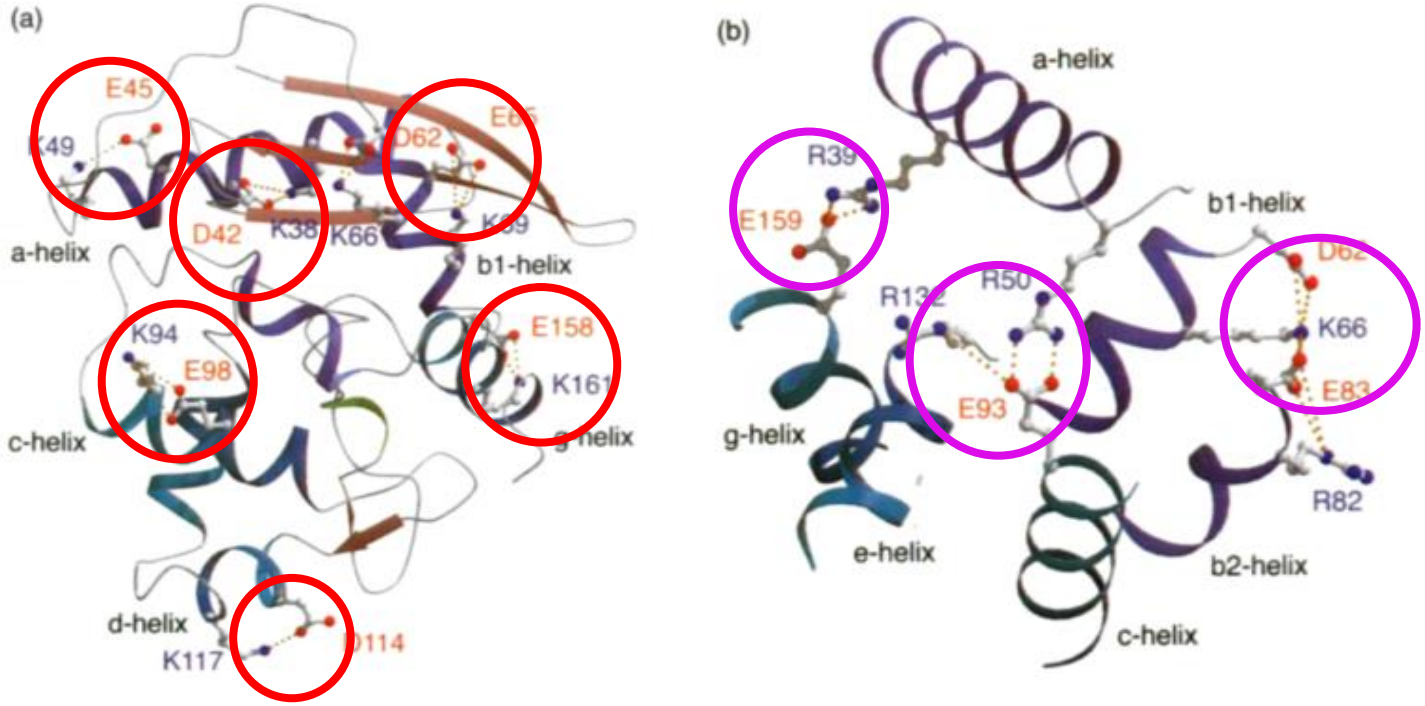
Table 1. Comparison of solvent-accessible surface areas

	Pk-MGMT	AdaC
Total solvent-accessible surface area (Å^2)	8160	8339
SASA of hydrophobic residues (Å^2) (% of total)	1935 (24)	2638 (32)
SASA of polar residues (Å^2) (% of total)	1797 (22)	2752 (33)
SASA of charged residues (Å^2) (% of total0	4428 (54)	2949 (35)
No. of residues in crystal structure	169	165
No. (% of total) of hydrophobic residues	75 (44.4)	78 (47.3)
No. (% of total) of polar residues	46 (27.2)	50 (30.3)
No. (% of total) of charged residues	48 (28.4)	37 (22.4)

Kai, *et al*. J. Mol. Biol. 1999, 292, 707

Ion pairs

Study on O^6-methylguanidine-DNA methyltransferase

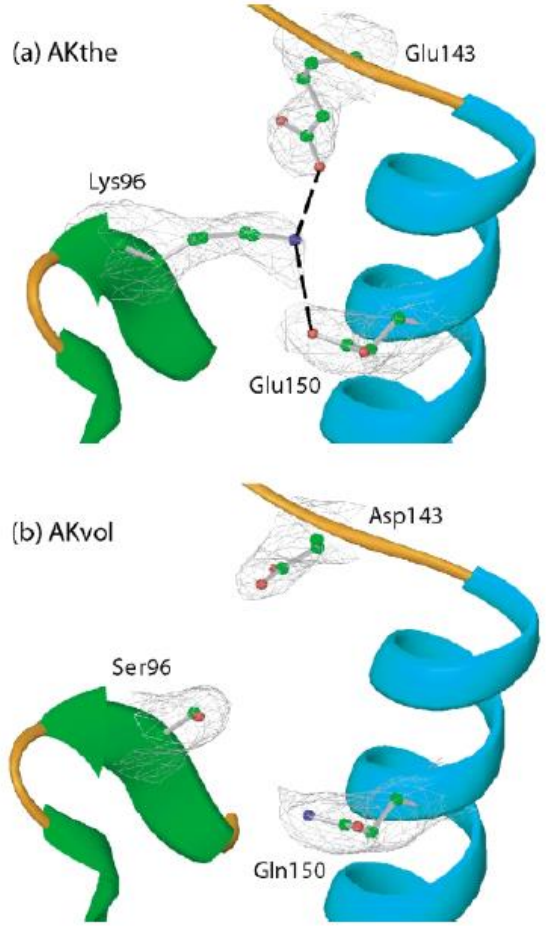


✔ Intrahelix/Interhelix ion pairs stabilize protein.

Kai, *et al*. J. Mol. Biol. 1999, 292, 707

Ion pairs

Study on adenylate kinases



Important for loop-stabilization

Philips *et al*, J. Mol. Biol. 2003, *330*, 1087

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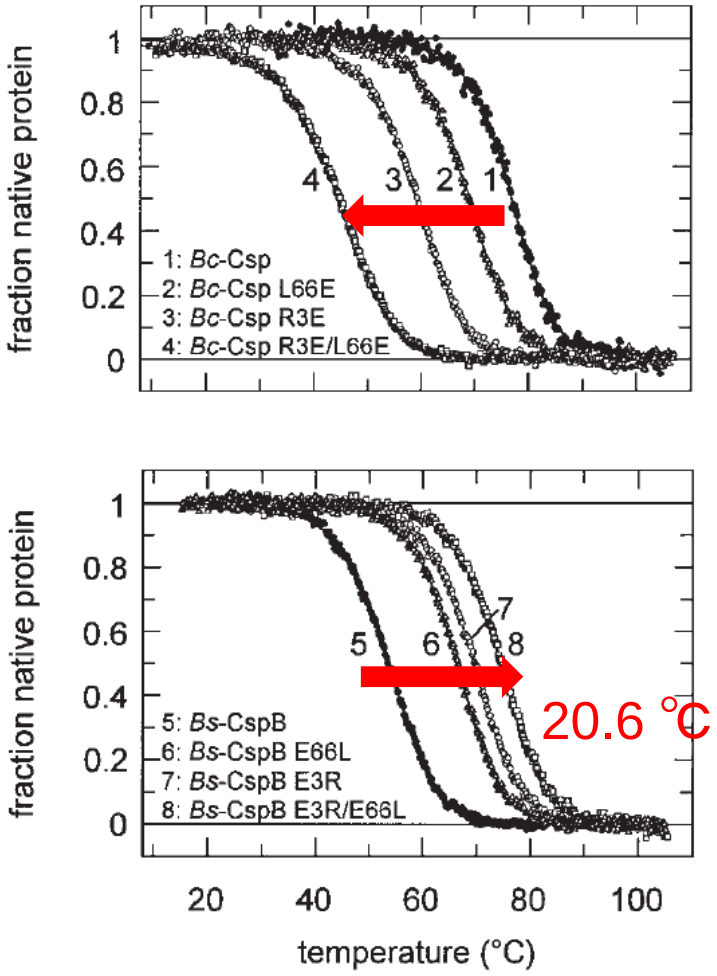
Improvement of stability by substitution



Bc-Csp: thermophilic

Bc-CspB: mesophilic

(Csp:cold shock protein)

Arg … electrostatic interaction

Leu … decreases the polarity around this residue to favor intramolecular hydrogen bond

Schmid, *et al*. Nat. Struct. Mol. Biol. 2000, 7, 380

Improvement of stability by substitution

Two methods

Direct Evolution Method

Rational Design Method

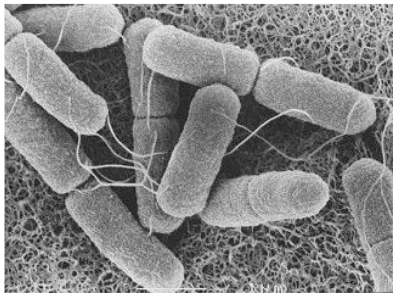
Direct evolution method

Cause evolution by “artificial selection”

selected gene used for next generation

Protein is extracted

Error-prone PCR
(2-3 bases are changed)



DNA is introduced to E. Coli

Direct evolution method

Study on subtilisin E (peptidase)

Table I. Thermostability of wild-type and evolved subtilisins E

Generation	Subtilisin E variant	dT_{50} (°C)	$t_{1/2}$ (min)			
			65°C	75°C	80°C	83°C
0	Wild-type	0	4.9			
	4A5	8.2	44.2			
	15C1	7.6	35.5			
1	32G11	2.3	9.8			
	35F10	7.8	39.5			
	36D10	3.1	15.2			
2	45B5	13.1	250	8.7		
	5H2	14.0	345	12.0		
3	16D11	14.1	368	12.8		
	20E8	14.2	391	13.6		
4	8B3	15.2	526	18.3	5.3	
5	3H5	17.2	1030		10.3	3.53

$t_{1/2}$… time that 50% of activity is lost at a fixed temperature
T_{50} … temperature that 50% of activity is lost during a fixed time

Zhao *et al*. Protein engineering. 1999, *12*, 47

Direct evolution method

Study on subtilisin E (peptidase)

Generation	Subtilisin E variant	Specfic activity (U/mg)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})
0	Wild-type	20.0	25.4	0.385	66.0
	4A5	40.0	52.4	0.388	135
	15C1	21.0	29.4	0.384	76.6
1	32G11	44.2	30.6	0.158	193
	35F10	40.6	52.3	0.383	136
	36D10	19.8	25.6	0.385	66.5
2	45B7	69.9	55.6	0.152	366
	5H2	71.6	58.8	0.155	379
3	16D11	73.0	57.1	0.150	381
	20E8	72.2	56.2	0.151	372
4	8B3	72.5	56.5	0.153	369
5	3H5	71.0	55.8	0.151	373

Activity increased.

Zhao *et al*. Protein engineering. 1999, *12*, 47

Direct evolution method

Study on subtilisin E (peptidase)

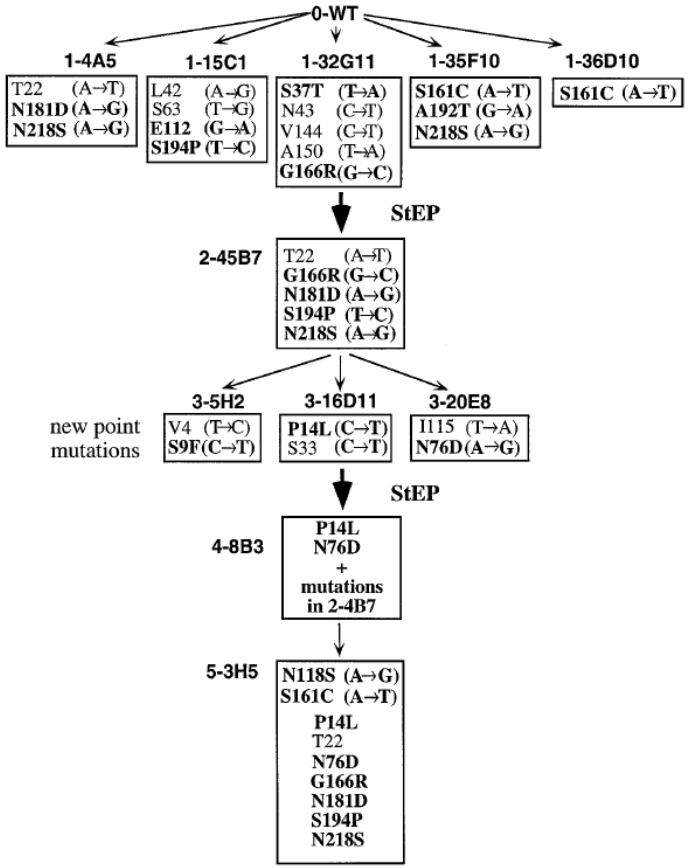


Fig. 4. Lineage and mutations in evolved thermostable subtilisin E variants. Base mutations are shown in parentheses.

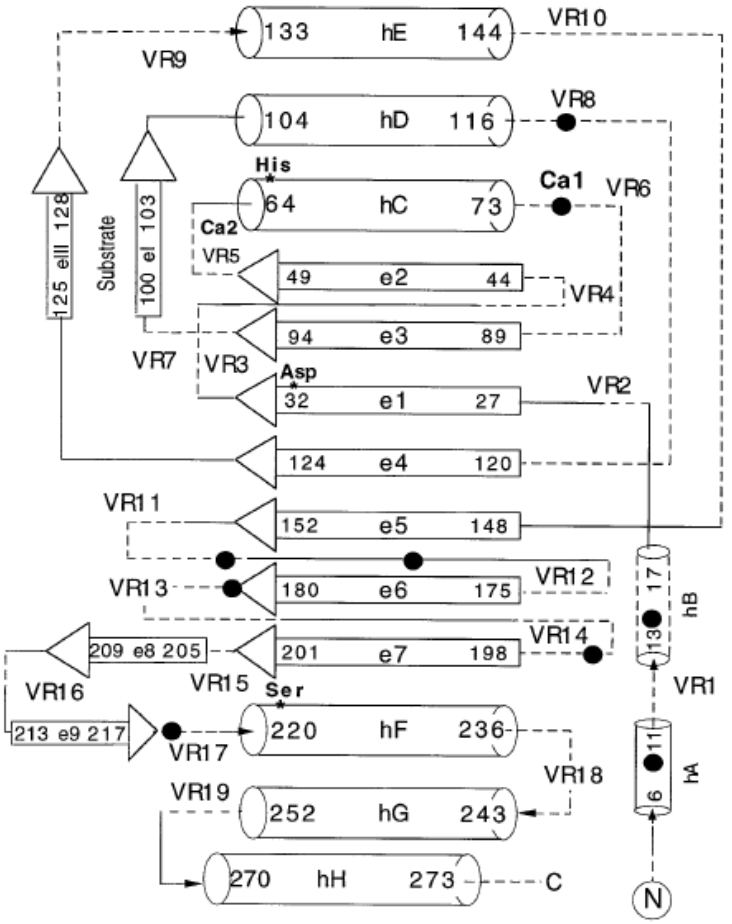
Analysis of mutation

Table III. Effects of amino acid substitutions on thermostability and activity (hydrolysis of s-AAPF-*p*NA) of subtilisin E

	Stability	Activity
S9F	+	0
P14L	+	0
S37T	0	0
N76D	+	0
N118S	+	0
S161C	+	0
G166R	+	+
N181D	+	0
A192T	0	0
S194P	+	(+)
N218S	+	+

Zhao *et al*. Protein engineering. 1999, *12*, 47

Direct evolution method



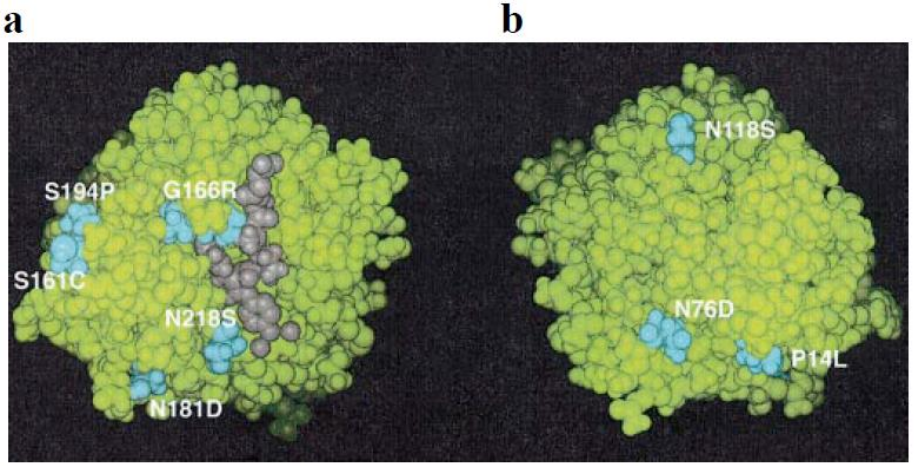


Fig. 6. Space-filling model of 5-3H5 subtilisin E showing the eight thermostabilizing mutations (cyan) and peptide substrate s-AAPF-*p*Na (gray). (**b**) View after rotation of (**a**) by 180°.

✔ Mutation at loop position.
✔ Mutated amino acids exist on the surface.

Zhao *et al*. Protein engineering. 1999, *12*, 47

Rational Design Method

Computer-based estimation of stability

Efficient screening is achieved.

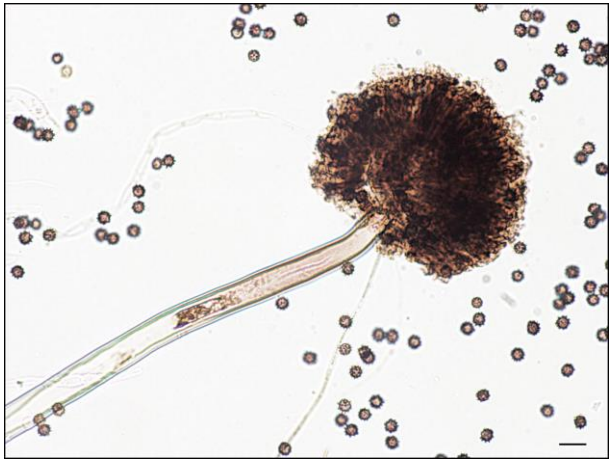
✔ Substitution candidates are estimated by calculation.

Rational Design Method

Study on L-rhamnosidase (r-Rha1)

Identified from *Aspergillus niger* (コウジカビ）

Increasing thermostability by substituting Lys to Arg. (constructing new hydrogen bonds and cation-π interactions)



García *et al*. Archives of Biochemistry and Biophysics. 2012, 528, 118

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

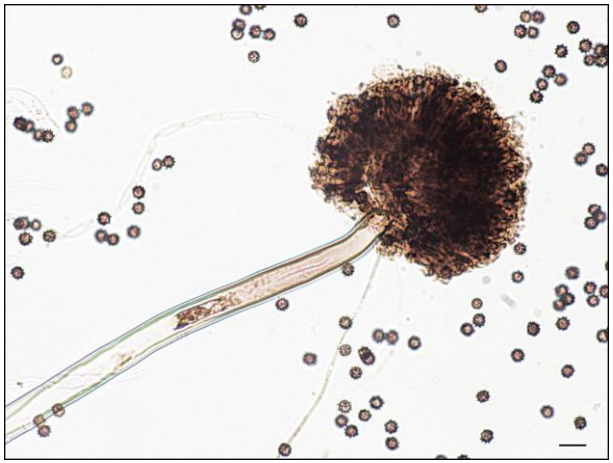
Rational Design Method

Study on L-rhamnosidase (r-Rha1)

Software for 3D structure … Modeller 9.15

for minimum energy … Minimization protocol

for site mutation … Mutation Energy protocol

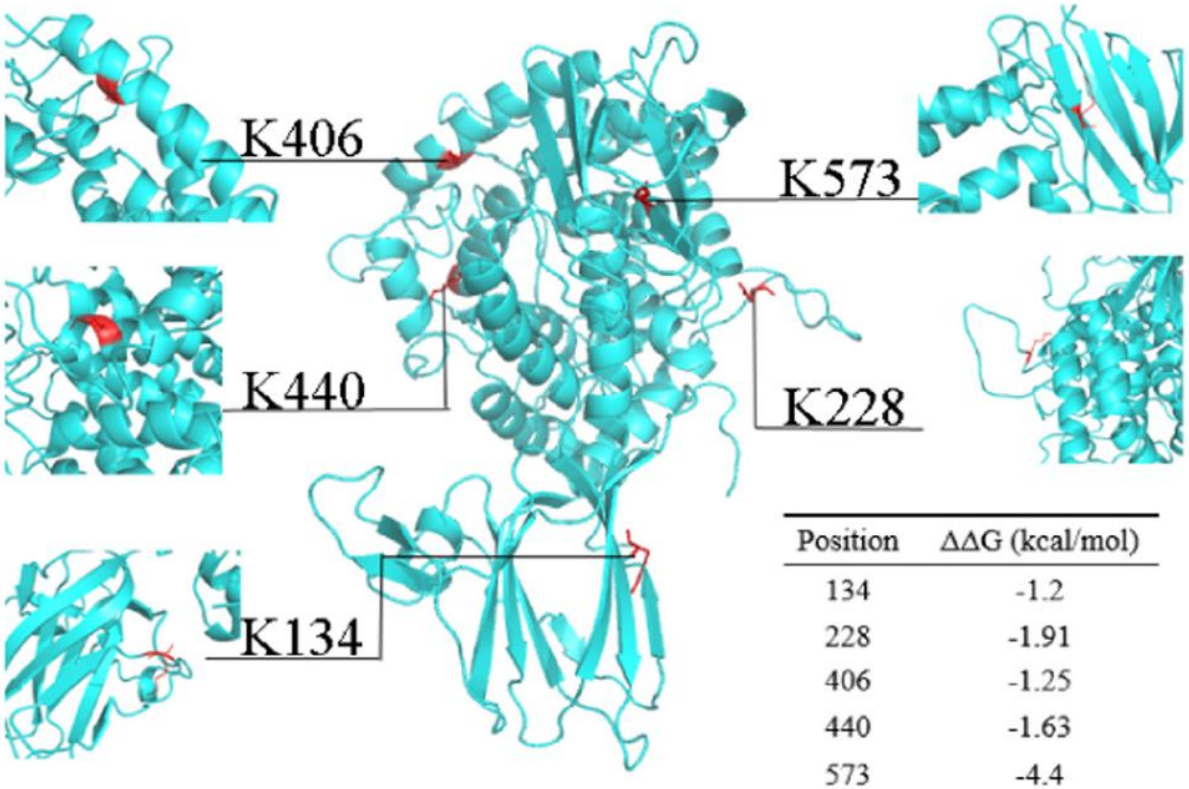


García *et al*. Archives of Biochemistry and Biophysics. 2012, 528, 118

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

Rational Design Method

The selected mutant positions as a result of calculation



Position	ΔΔG (kcal/mol)
134	-1.2
228	-1.91
406	-1.25
440	-1.63
573	-4.4

Fig. 1. The selected mutant positions and ΔΔG values of 134, 228, 406, 440 and 573. The diagram showed a structural model based on comparative protein modeling using Modeller 9.15. The table in the lower right corner showed the values of the predicted Gibbs free energy changes (ΔΔG) for the substitution of Lys with Arg on these positions.

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

Rational Design Method

Stability and activity of single mutants

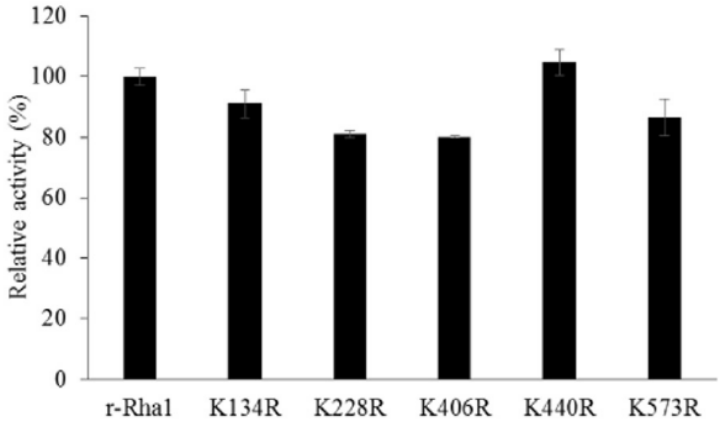


Fig. 2. The relative activities of five single mutants compared to the wild type r-Rha1.

Table 1
Enzymatic properties of r-Rha1 and single-site mutants.

Samples	T_{opt} (°C)	pH_{opt}	$t_{1/2}$		
			60 °C (h)	65 °C (min)	70 °C (min)
r-Rha1	60	4	8.0	39	4.0
K134R	60	4	<2.0	6.5	2.6
K228R	60	5	6.0	34	4.2
K406R	60	5	10	50	4.6
K440R	60	5	8.5	50	5.8
K573R	60	4	8.3	49	5.0

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

Rational Design Method

Stability and activity of single/double mutants

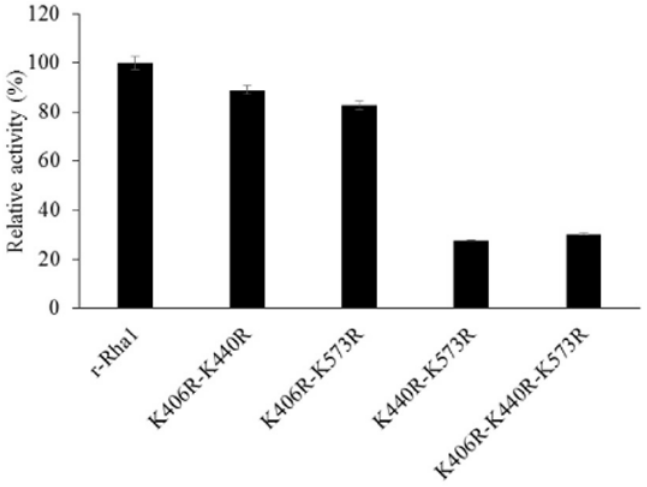


Fig. 3. The relative activities of the wild type r-Rha1 and the multiple-site mutant enzymes.

Table 2
Enzymatic properties of r-Rha1 and combinational mutants.

Samples	T_{opt} (°C)	pH_{opt}	$t_{1/2}$		
			60 °C (h)	65 °C (min)	70 °C (min)
r-Rha1	60	4	8.0	39	4.0
K406R-K440R	60	4	9.6	60	6.8
K406R-K573R	60	4	11	62	7.5

Table 3
Kinetic parameters of r-Rha1 and combinational mutants.

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Wild-type	10.779	1.741×10^4	1.615×10^3
K406R-K440R	12.158	1.415×10^4	1.164×10^3
K406R-K573R	23.083	7.302×10^3	3.163×10^2

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

Rational Design Method

Analysis

For destabilized mutants

Hydrophobic interactions may be hindered.

For K406R-K440R and K406R-K573R

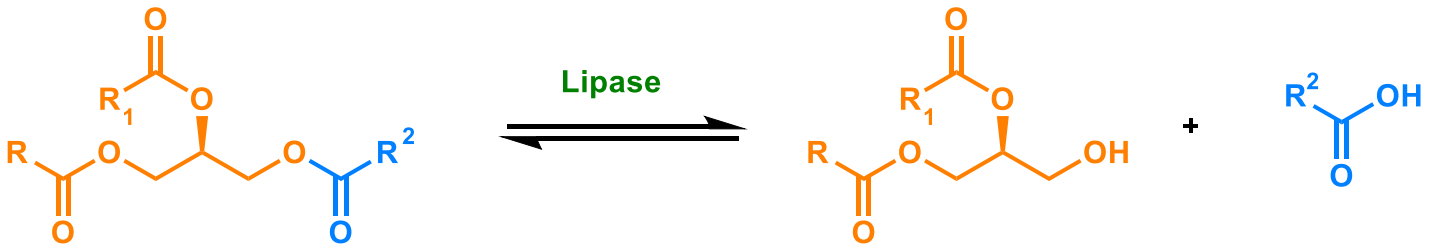
No extra intramolecular interactions, compared to single mutants. May be due to the compactness of the protein.

Ni, *et al*. Int. J. Biol. Macromol. 2018, *112*, 14

Rational Design Method

Study on *Candida rugose* lipases (CRL)

- Lipase can catalyze hydrolysis and esterification.



- Starts inactivation at 45 ℃

Xu, Yan, *et al*. RSC Adv. 2018, *8*, 1948

Study on the protein stability

Three prediction algorithms were used.

- FoldX … estimates Gibbs energy of protein folding from empirical data.
- Rosetta ddg_monomer … predicts mutation-induced stability change.
- I-Mutant3.0 … predicts stability of mutants from database

Xu, Yan, *et al*. RSC Adv. 2018, *8*, 1948

Study on the protein stability

Result of estimation

Table 1 Stable mutants identified by all three computational methods and correspondent estimated value

Mutants	FoldX[a] (kcal mol^{-1})	Rosetta ddg_monomer[a] (kcal mol^{-1})	I-Mutant 3.0[b] (kcal mol^{-1})
Asp457Phe	−6.08	−12.41	0.09
Asp457Leu	−4.73	−4.71	0.47
Asp457Met	−4.95	−5.15	0.15
Asp457Tyr	−4.99	−11.96	0.07
Asp457Trp	−4.21	−10.03	0.18
Glu66Phe	−2.63	−3.07	0.32

[a] Rosetta ddg_monomer returns negative value for stabilizing mutations. The greater the absolute value of negative numbers, the greater likelyhood to be more stable. [b] Positive values predicted by I-Mutant 3.0 indicate that induced mutations are more stable.

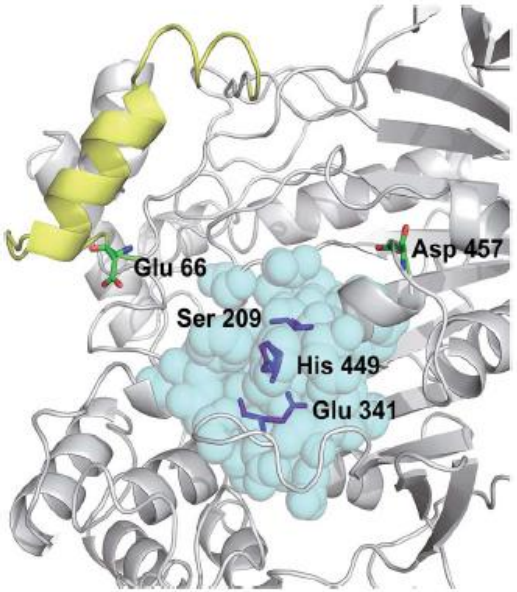


Fig. 1 Positions of mutated residues. Catalytic triad, magenta sticks; residues around 5 Å of the catalytic triad, cyan balls; lid structure, yellow. Positions of Glu66 and Asp457 are also shown.

Glu66… important for the activity
Asp457… at loop structure.

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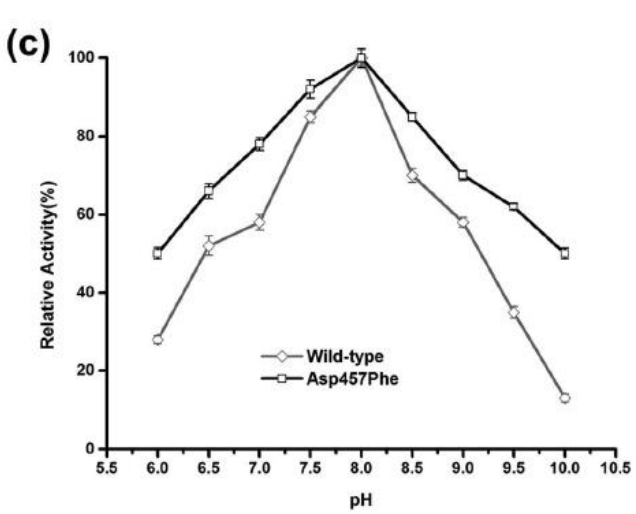
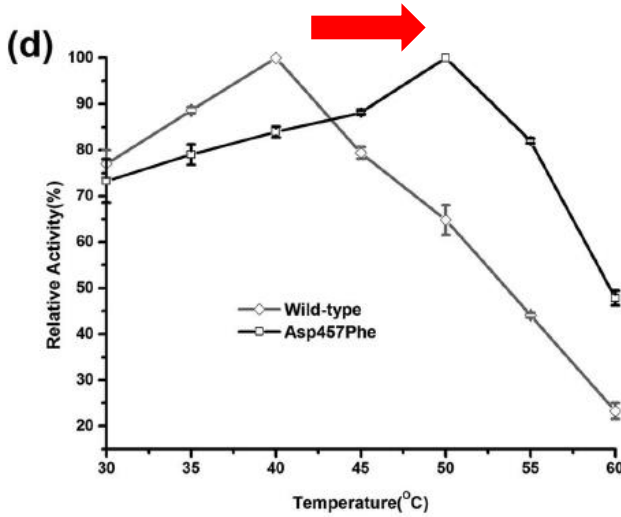
Results

Mutants	ΔT_m (°C)
Wild-type	0
Asp457Leu	8.4
Asp457Met	7.4
Asp457Trp	8.9
Asp457Tyr	8.4
Asp457Phe	9.3





- ✔ Asp457Phe was most effective
- ✔ Higher stability to acidic/alkaline condition
- ✔ Higher optimal temperature achieved

Xu, Yan, *et al*. RSC Adv. 2018, *8*, 1948

Comparison of kinetic parameters

Enzyme	T_{opt} (°C)	K_m[a] (μM)	k_{cat}[a] (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})
WT	40	8.5 ± 0.8	4006 ± 127	473 ± 30
Asp457Phe	50	9.2 ± 0.7	4234 ± 151	461 ± 19

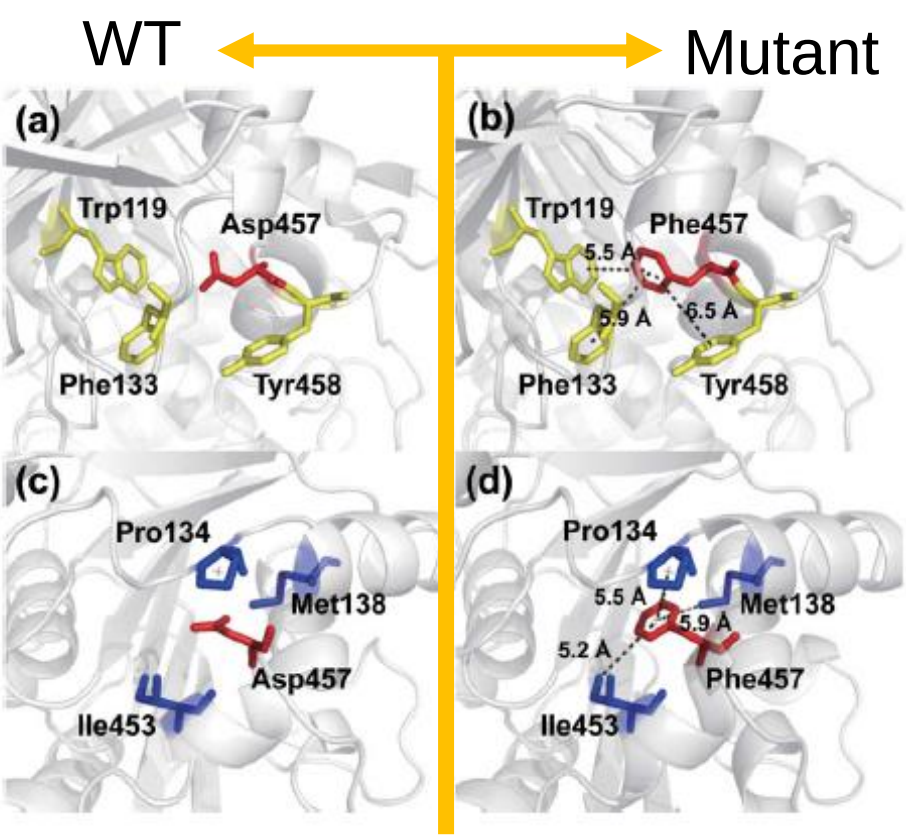
✔ Kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) of the mutant was inferior to WT, but it's negligible.

✔ What is the origin of stability???

Xu, Yan, *et al*. RSC Adv. 2018, *8*, 1948

Analysis

Protein models were constructed by SWISS.



✔ aromatic interaction network and hydrophobic interaction

✔ Hydrophobic interaction increases pH tolerance, for it decreases SASA (solvent accessible surface area).

Xu, Yan, *et al*. RSC Adv. 2018, *8*, 1948

Summary

- The origin of protein thermostability is derived from several factors.
- Thermostability can be increased by the single or several substitution of amino acids.
- Thermostability can be increased by artificial amino acid substitution.

Appendix

Deamidation of proteins

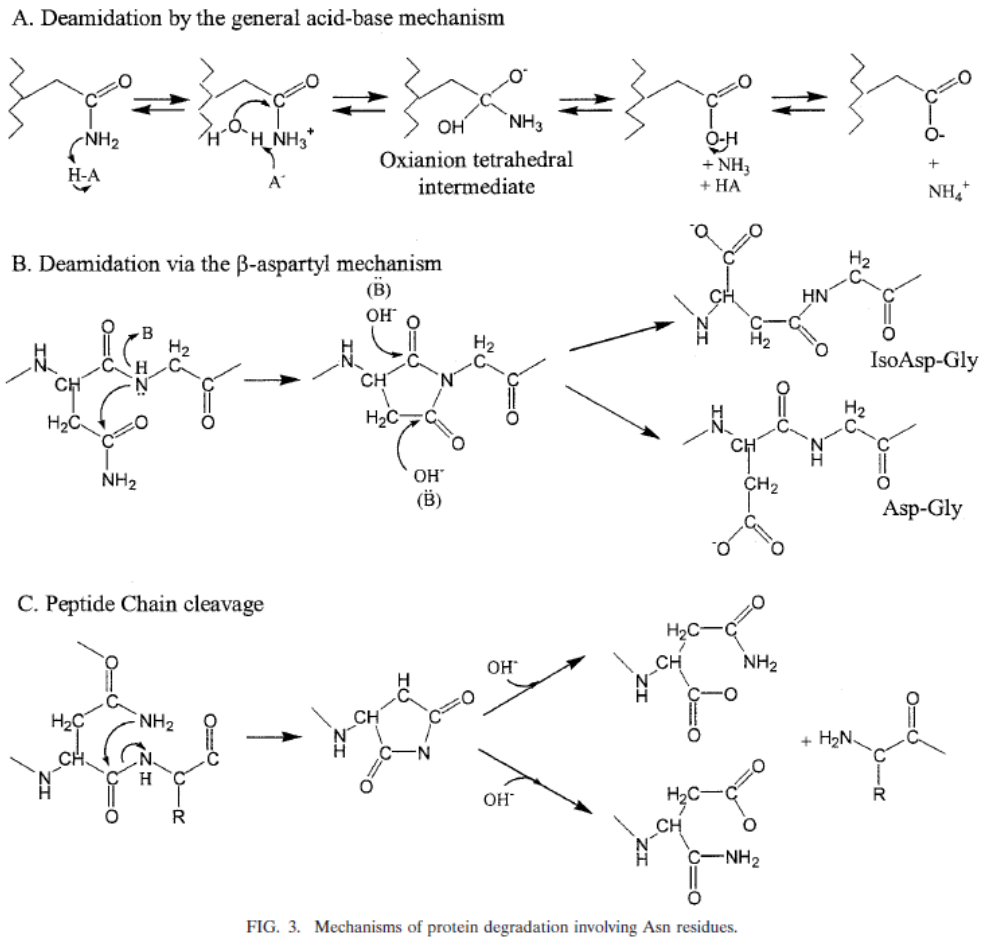


FIG. 3. Mechanisms of protein degradation involving Asn residues.

These reactions are prohibited so far as protein is folded correctly.

Hensel *et al*. Biochem. Soc. Symp. 1992, 58, 127

Zeikus *et al*. MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS. 2001, 65, 1