



TOHOKU  
UNIVERSITY

# ***Aspergillus oryzae* is good at usage of hydrolyzing enzyme on solid-surfaces**

— Recruitment of polyesterase (cutinase) of *A. oryzae* by the biosurfactant protein hydrophobin RoIA on plastics



日本酒  
(Sake)



醤油  
(soy sauce)

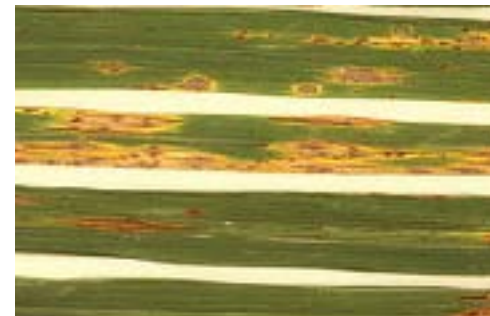
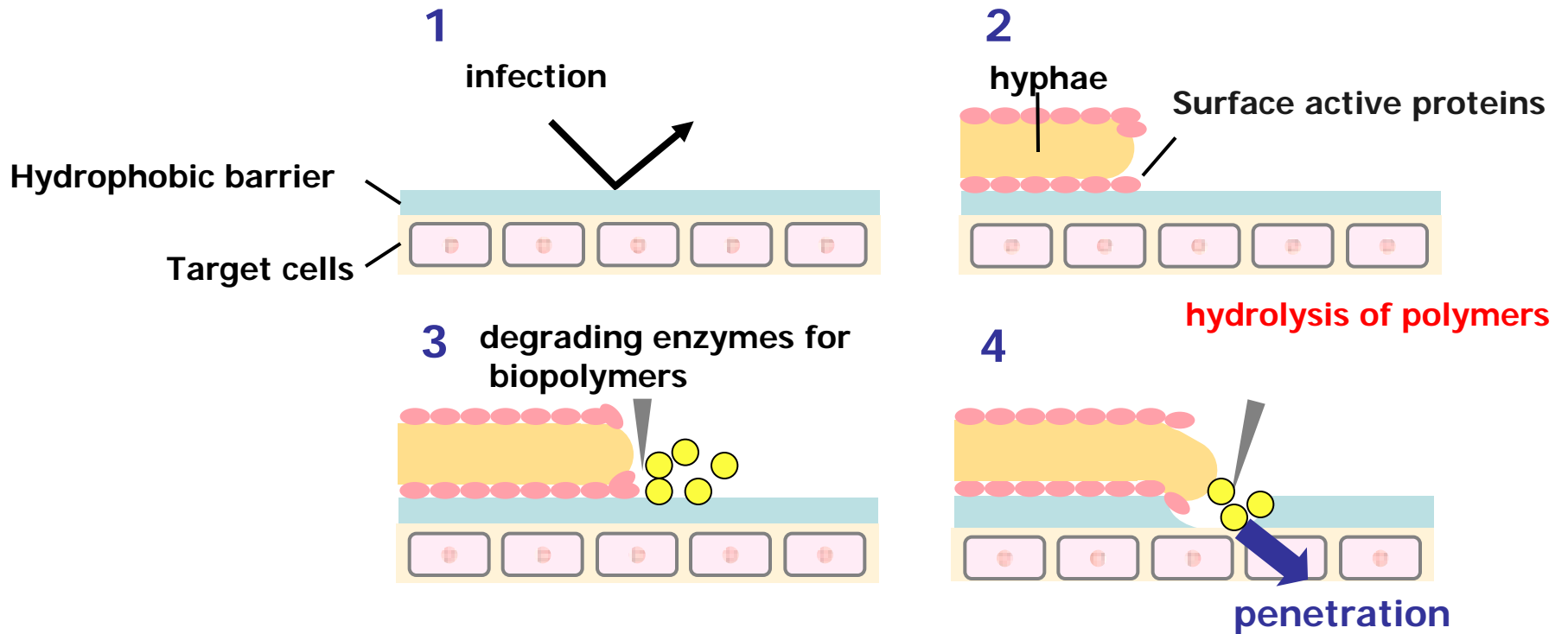
**\*Keietsu Abe<sup>1,2</sup>, Toru Takahashi<sup>1</sup>**

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# TOPICS

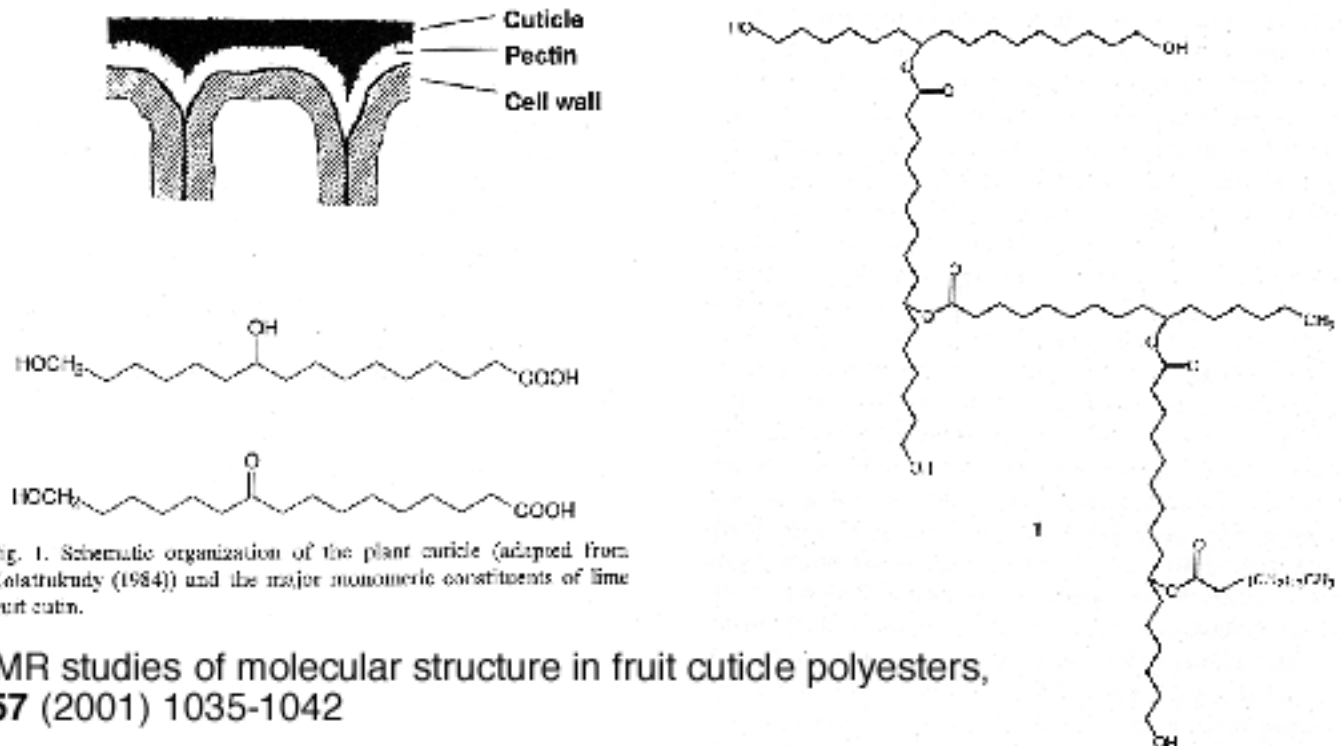
- *Aspergillus oryzae* and Biodegradable Plastic Recycle System
  - Process design
  - Plastic-degrading enzyme CutL1 and a novel plastic-degrading factor hydrophobin RoIA
- Novel Molecular Functions of RoIA
  - Recruitment of soluble esterase CutL1 by RoIA attached to hydrophobic surfaces

# Fungi degrade biopolymers in nature



pathogens

# PBSA mimics wax polyesters



X. Fang, *et al.*, NMR studies of molecular structure in fruit cuticle polyesters, *Phytochemistry*, **57** (2001) 1035-1042

- **Surface materials of plants**
- **Wax Polyesters (= biodegradable)**

10 million t /year in Japan

**Synthetic  
Plastics**

**incineration**

**CO<sub>2</sub>, Dioxin  
Pollution**

Shift

**Capacity?  
Time?  
Cost recovery?**

**Degradation in Soil**

**Biodegradable Plastics**

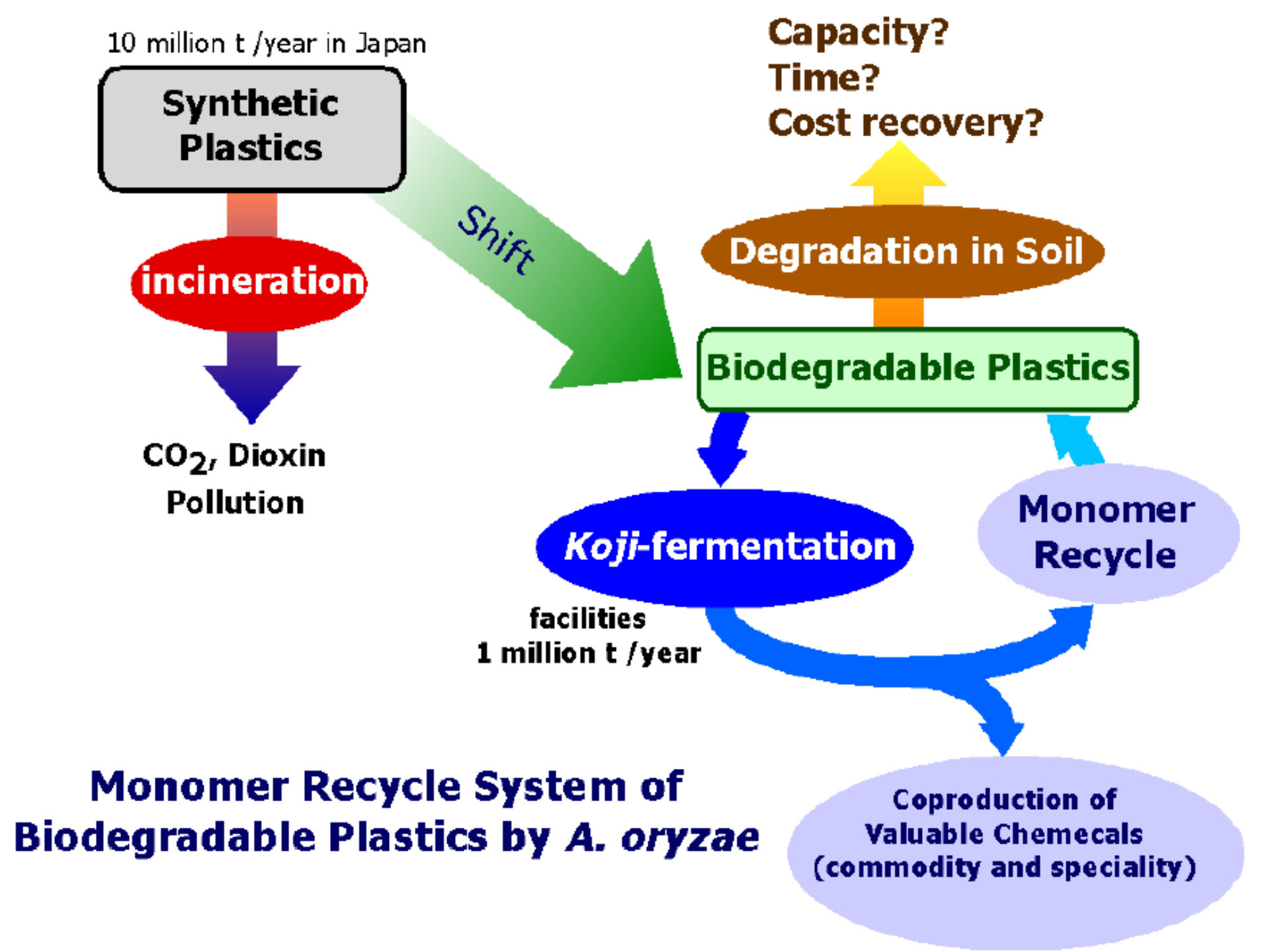
**Koji-fermentation**

**facilities  
1 million t /year**

**Monomer  
Recycle**

**Coproduction of  
Valuable Chemecals  
(commodity and speciality)**

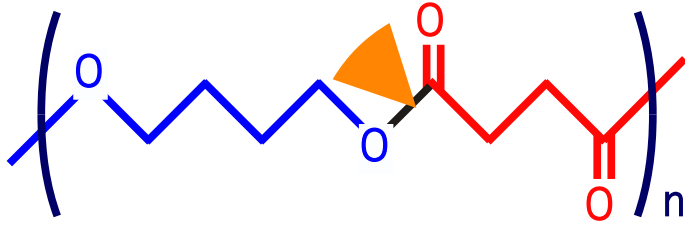
**Monomer Recycle System of  
Biodegradable Plastics by *A. oryzae***



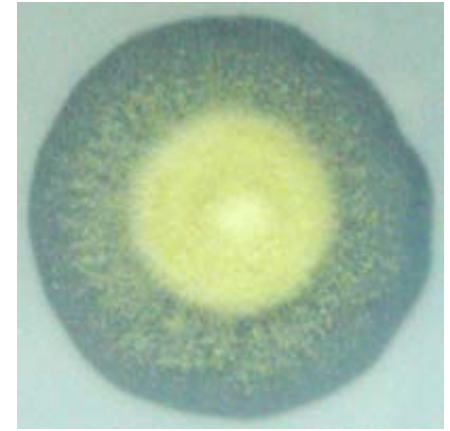
# PBSA recycling system mimics koji-fermentation



# PBSA degradation by *A. oryzae* RIB40



PBSA ; polybutylene succinate co-adipate



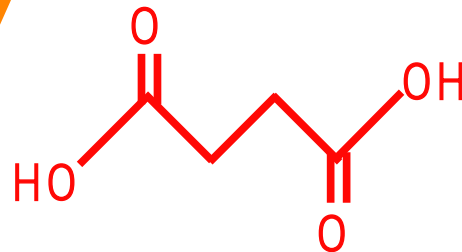
PBSA-CD Agar Plate

degradation by cutinase (CutL1)

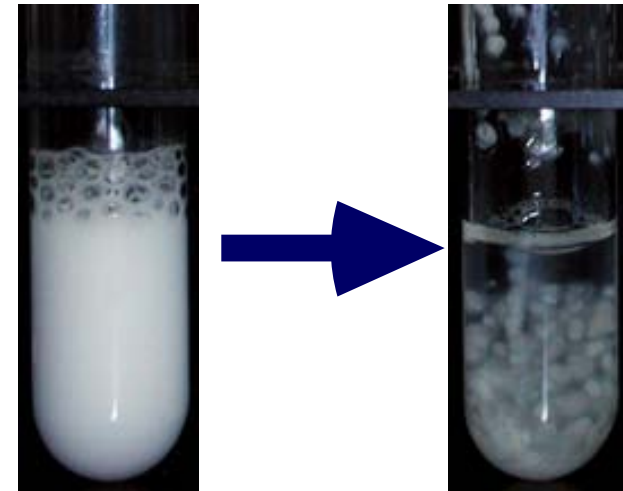
(Maeda *et al.* 2005; AMB)



1,4 - butanediol

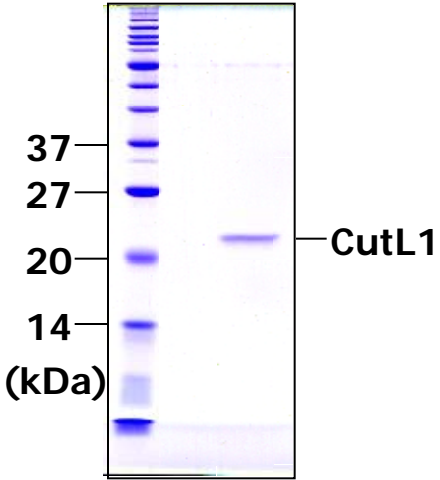


succinate

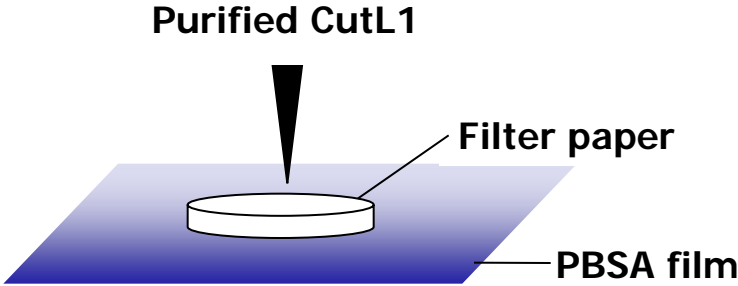


PBSA-CD Liquid Medium

# CutL1, PBSA degrading esterase produced by *A. oryzae*

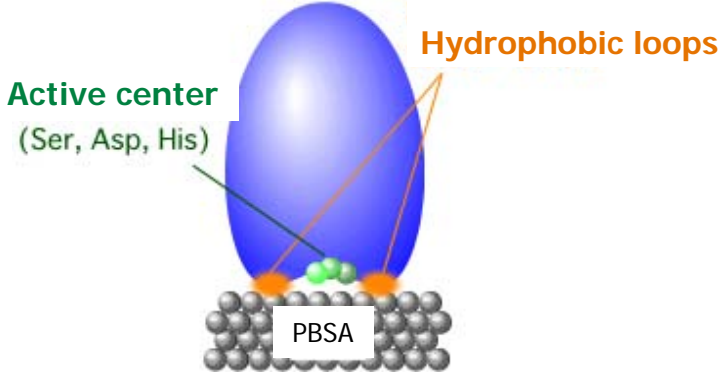
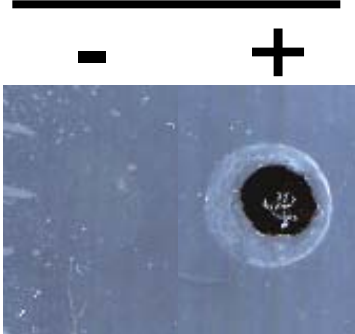


SDS-PAGE analysis of purified CutL1



Incubation at 37 °C for 6 hours  
Removal of the filter paper

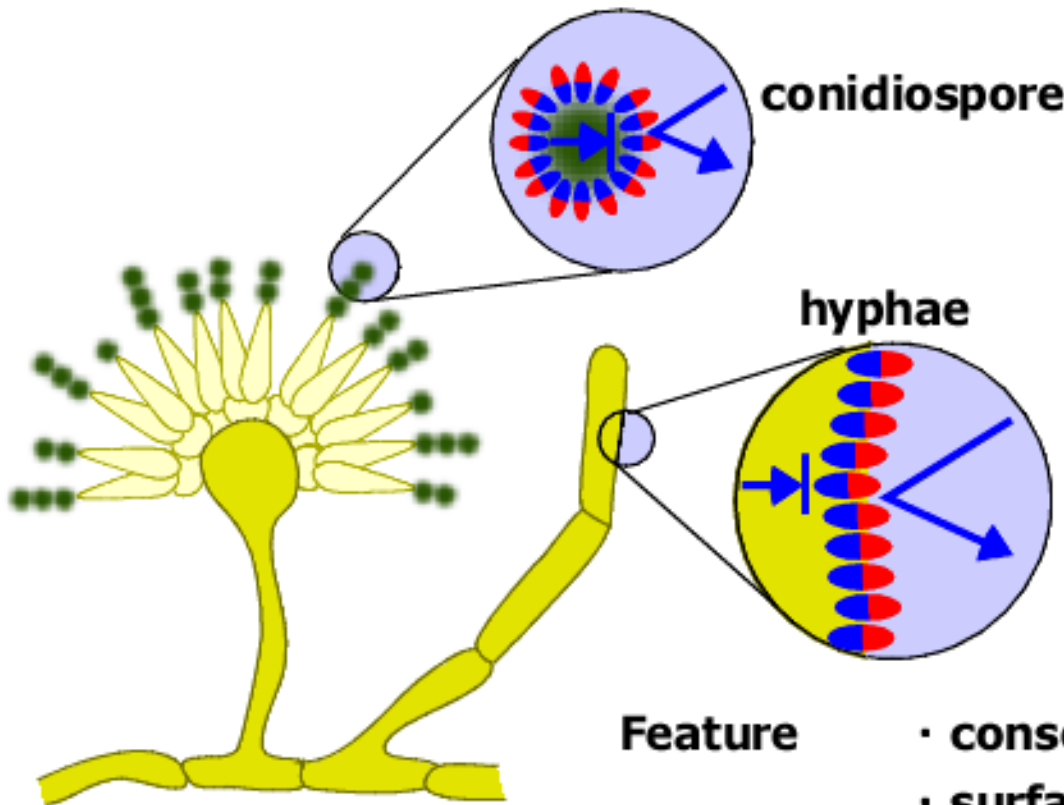
CutL1



The schematic model of PBSA-degradation by CutL1

Degradation of a PBSA film by CutL1

# What is hydrophobin?



Hydrophilic Hydrophobic



Biosurfactant

Feature

- conserved 8 cysteine residues
- surface active

Localization

- aerial hyphae
- outer - Hydrophobic phase
- no expression in liquid culture

Function

- protection layer
- resistant to drying

# Alignment of deduced amino acid sequences of RoIA homologs

<i>Aspergillus oryzae</i> RoIA	1	M	QFS	-	VA	VL	AL	AT	VA	AL	PP	A-	SG	TG	AG	QQ	VGH	-	SK	-	N	-	DF	PL	PK	EL	T	44																							
<i>Aspergillus fumigatus</i> Hyp1	1	M	KF	SL	SA	AV	LA	FA	VS	VA	AL	-	PQ	HD	VA	AG	NG	VGN	KG	NA	NV	RF	VP	DD	IT	48																									
<i>Aspergillus nidulans</i> RodA	1	M	KF	SI	AA	VV	FA	AA	SV	AA	LP	PA	HD	SQ	AG	NG	VGN	KG	NS	NV	KF	VP	EN	VT	49																										
<i>Lentinula edodes</i> Hyd2	1	M	QF	-	-	-	-	KL	-	AF	-	VSI	-	AL	-	-	-	-	-	-	A	-	TL	A	-	-	VAT	-	P	-	AP	RGE	25																		
<i>Flammulina velutipes</i> Fvh1	1	M	VS	F	-	-	-	R	-	AF	TVA	-	ASL	-	-	-	-	-	-	FA	-	TL	A	-	-	AAT	-	P	-	LDT	AL	27																			
<i>Aspergillus oryzae</i> RoIA	45	T	KQ	AD	K	G	DQ	AQ	L	T	C	C	N	K	T	V	K	T	G	D	F	T	Q	V	E	E	G	L	X	A	G	L	L	S	N	L	L	G	A	G	Q	G	S	Q	G	94					
<i>Aspergillus fumigatus</i> Hyp1	49	V	KQ	AT	E	K	C	E	DQ	AQ	L	S	C	C	N	K	A	T	Y	A	G	D	V	T	D	I	D	E	G	I	L	A	G	T	L	K	N	L	I	G	G	G	S	G	T	E	G	98			
<i>Aspergillus nidulans</i> RodA	50	V	KQ	AS	D	K	C	G	DQ	AQ	L	S	C	C	N	K	A	T	Y	A	G	D	T	T	T	V	D	E	G	L	L	S	G	A	L	S	G	L	I	G	A	G	S	G	A	E	G	99			
<i>Lentinula edodes</i> Hyd2	26	P	-	-	A	S	S	-	C	-	S	T	G	L	Q	C	C	N	-	T	V	E	P	A	-	-	S	S	P	S	A	S	T	I	L	G	L	L	G	I	V	-	I	Q	G	V	D	V	L	-	66
<i>Flammulina velutipes</i> Fvh1	28	P	R	-	A	A	D	Q	C	-	N	V	S	N	Q	C	C	N	-	S	V	Q	Q	A	-	-	S	S	G	P	A	A	L	I	L	G	L	L	G	V	-	L	Q	D	V	N	V	L	-	70	
<i>Aspergillus oryzae</i> RoIA	95	L	G	L	D	E	C	T	N	I	-	P	V	I	P	-	I	-	I	S	I	A	S	-	P	Q	E	K	C	K	Q	P	I	S	C	C	Q	N	T	K	S	A	D	G	L	V	G	I	140		
<i>Aspergillus fumigatus</i> Hyp1	99	L	G	L	F	N	Q	C	S	N	V	L	Q	I	P	V	I	G	I	P	I	Q	A	L	V	N	Q	K	C	K	Q	N	I	A	C	C	Q	N	S	P	S	D	A	S	G	L	I	G	L	148	
<i>Aspergillus nidulans</i> RodA	100	L	G	L	F	D	Q	C	S	K	L	D	V	A	V	-	L	I	G	-	I	Q	D	L	V	N	Q	K	C	K	Q	N	I	A	C	C	Q	N	S	P	S	A	D	G	N	L	I	G	V	146	
<i>Lentinula edodes</i> Hyd2	67	V	G	L	-	-	T	C	-	-	-	-	-	-	SP	-	I	T	V	-	I	G	L	E	S	G	-	C	S	A	Q	A	V	C	C	-	-	T	D	N	-	S	N	G	L	I	S	I	102		
<i>Flammulina velutipes</i> Fvh1	71	V	G	L	-	-	D	C	-	-	-	-	-	-	SP	-	I	T	V	-	I	G	G	G	N	G	-	C	N	A	S	P	V	C	C	-	-	E	N	N	-	S	F	G	S	L	I	S	I	106	
<i>Aspergillus oryzae</i> RoIA	141	G	L	P	C	I	A	L	G	S	L	151																																							
<i>Aspergillus fumigatus</i> Hyp1	149	G	L	P	C	I	A	L	G	S	I	L	159																																						
<i>Aspergillus nidulans</i> RodA	147	G	L	P	C	V	A	L	G	S	I	L	157																																						
<i>Lentinula edodes</i> Hyd2	103	G	-	-	C	L	P	-	-	V	T	L	109																																						
<i>Flammulina velutipes</i> Fvh1	107	G	-	-	C	V	P	-	-	I	S	I	113																																						

## **PBSA degradation by recombinant *A.oryzae***

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<b>Strains</b>	<b>Degradation ratio (%)</b>
<i>A.oryzae</i> wt	7
RolA overexpressing strain	25
CutL1 overexpressing strain	61
<b>CutL1, RolA co-overexpressing strain</b>	<b>71</b>

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# TOPICS

- *Aspergillus oryzae* and Biodegradable Plastic Recycle System
  - Process design
  - Plastic-degrading enzyme CutL1 and a novel plastic-degrading factor hydrophobin RoIA
- **Novel Molecular Functions of RoIA**
  - Recruitment of soluble esterase CutL1 by RoIA attached to hydrophobic surfaces

# Purification of RoIA from culture broth of *A. oryzae* overexpressing RoIA

## RoIA overexpressing *A.oryzae* conidiospore suspension

- inoculated ( $1 \times 10^6$  spore per ml) to CD medium (1 % Fructose)
- cultured at 30°C for 48 hr
- filtrated with MIRACLOTH

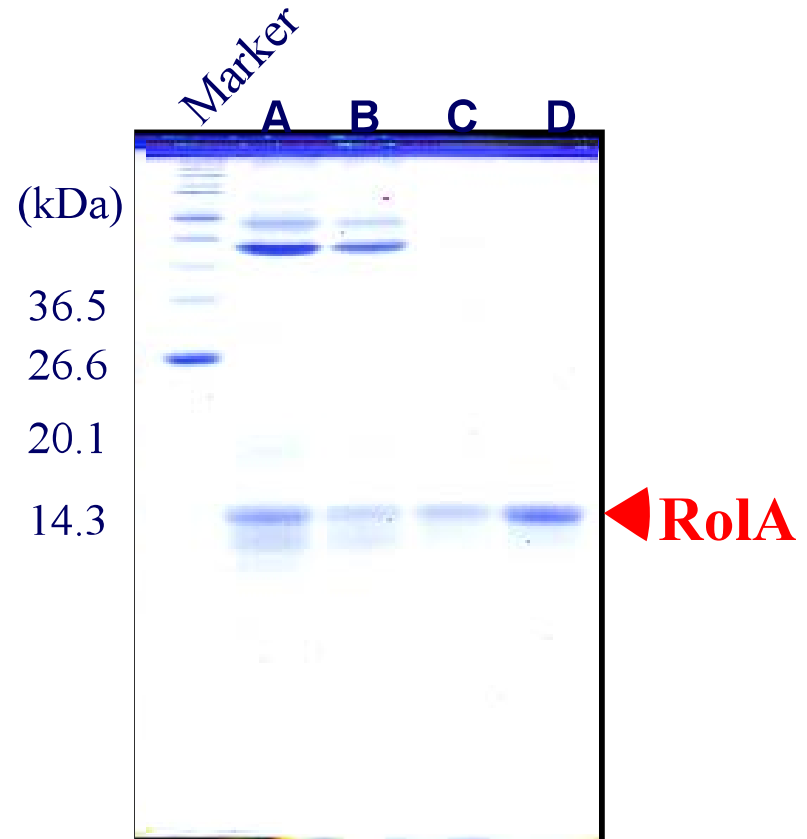
## Culture broth; **A**

- added solid  $(\text{NH}_4)_2\text{SO}_4$  to 40 % saturation; **B**
- centrifuged 12000 *g*, 4°C, 20 min

## Supernatant

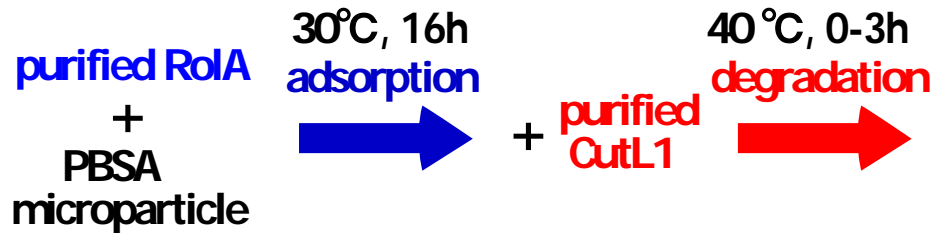
- Octyl-Cellulofine type S column; **C**
- S-Sepharose FF column; **D**

## Purified RoIA

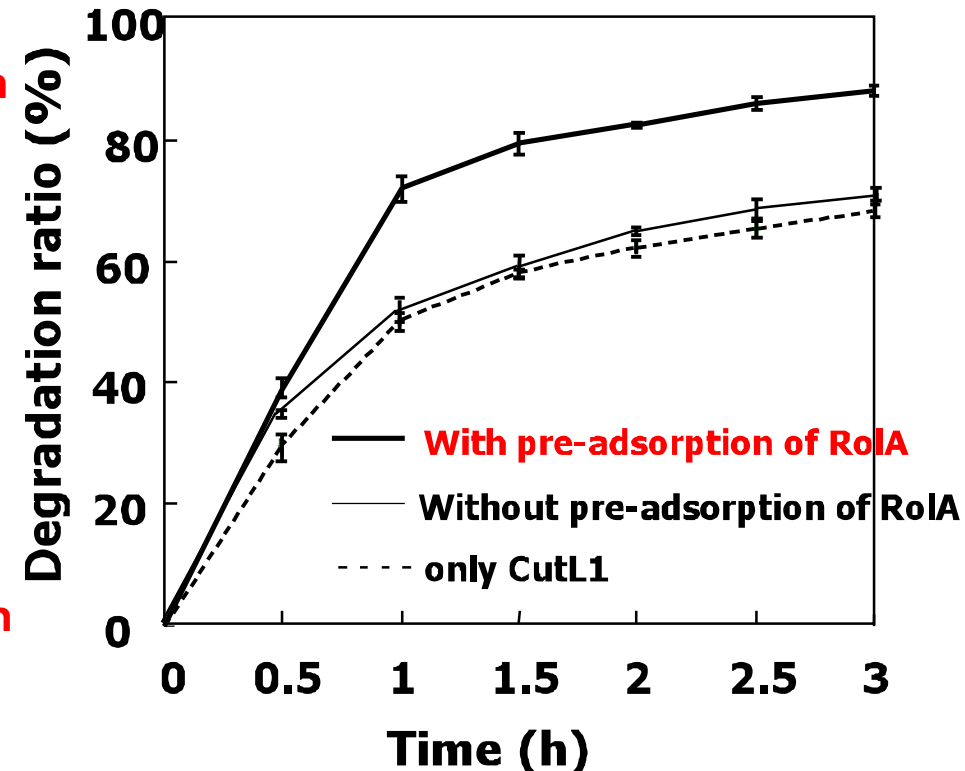
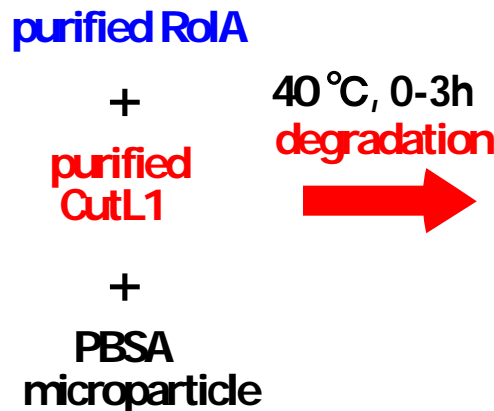


# Effect of RoIA on PBSA degradation by CutL1

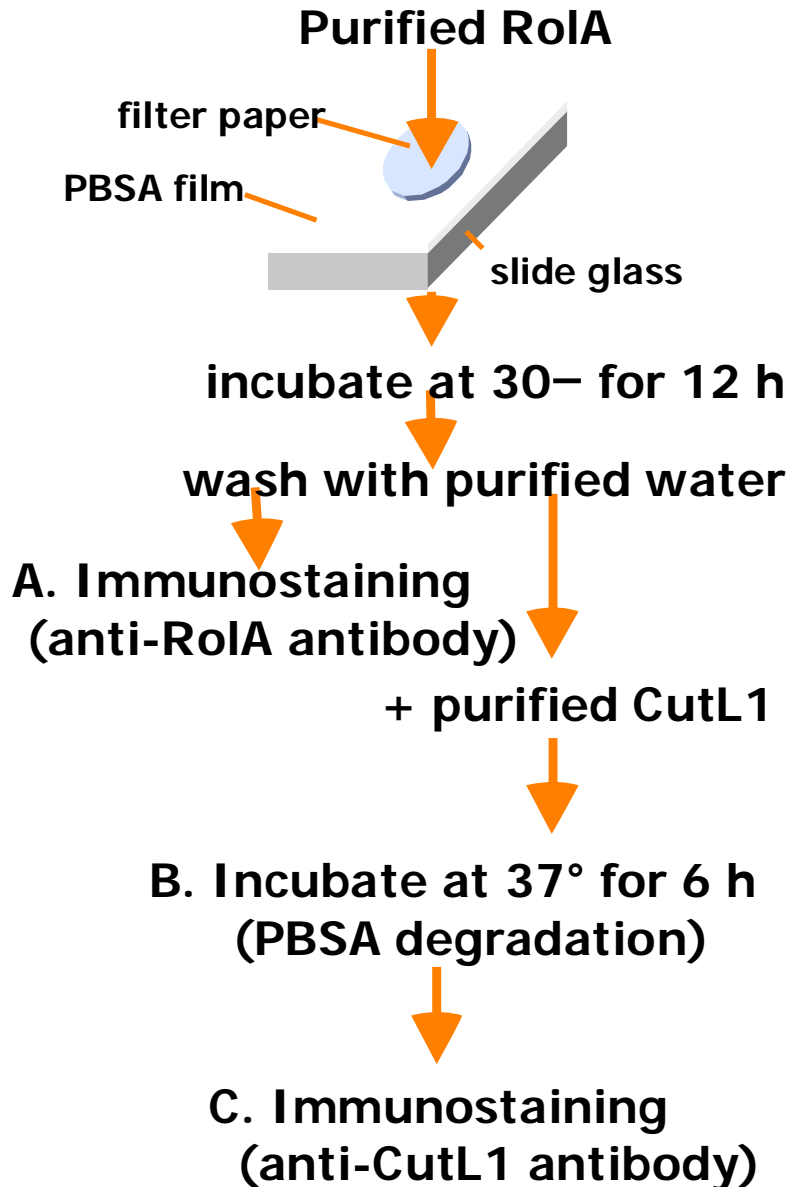
## With pre-adsorption of RoIA



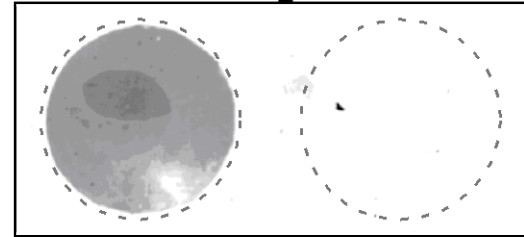
## Without pre-adsorption of RoIA



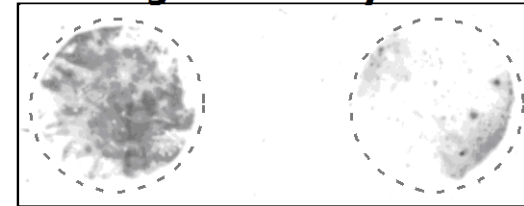
# Adsorption of CutL1 onto a PBSA film pre-coated with RoIA



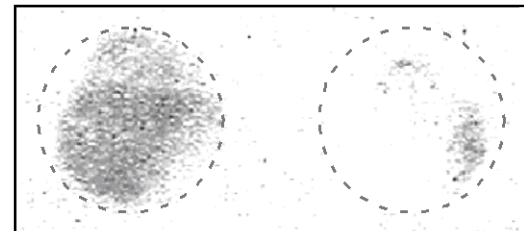
## A. Immunostaining with anti-RoIA antibody



## B. PBSA degradation by CutL1



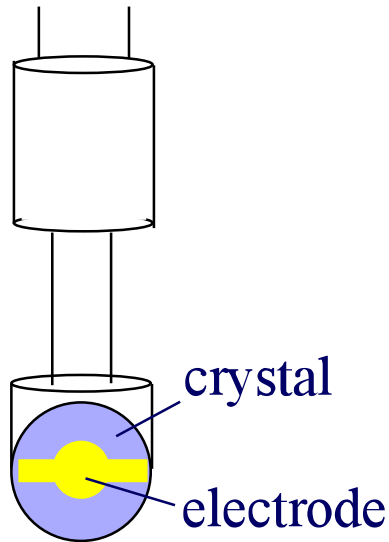
## C. Immunostaining with anti-CutL1 antibody



+ RoIA  
pre-coat

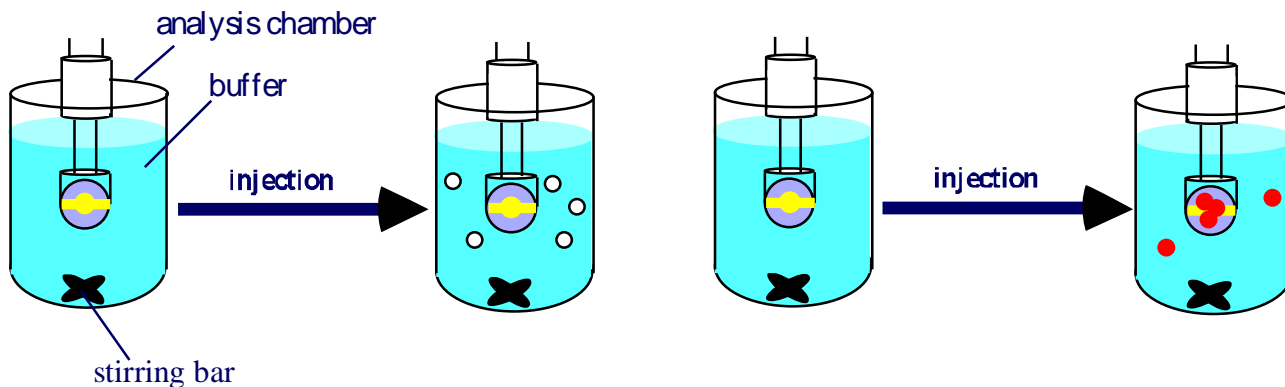
- RoIA  
pre-coat

# Molecular Interaction Analysis by QCM

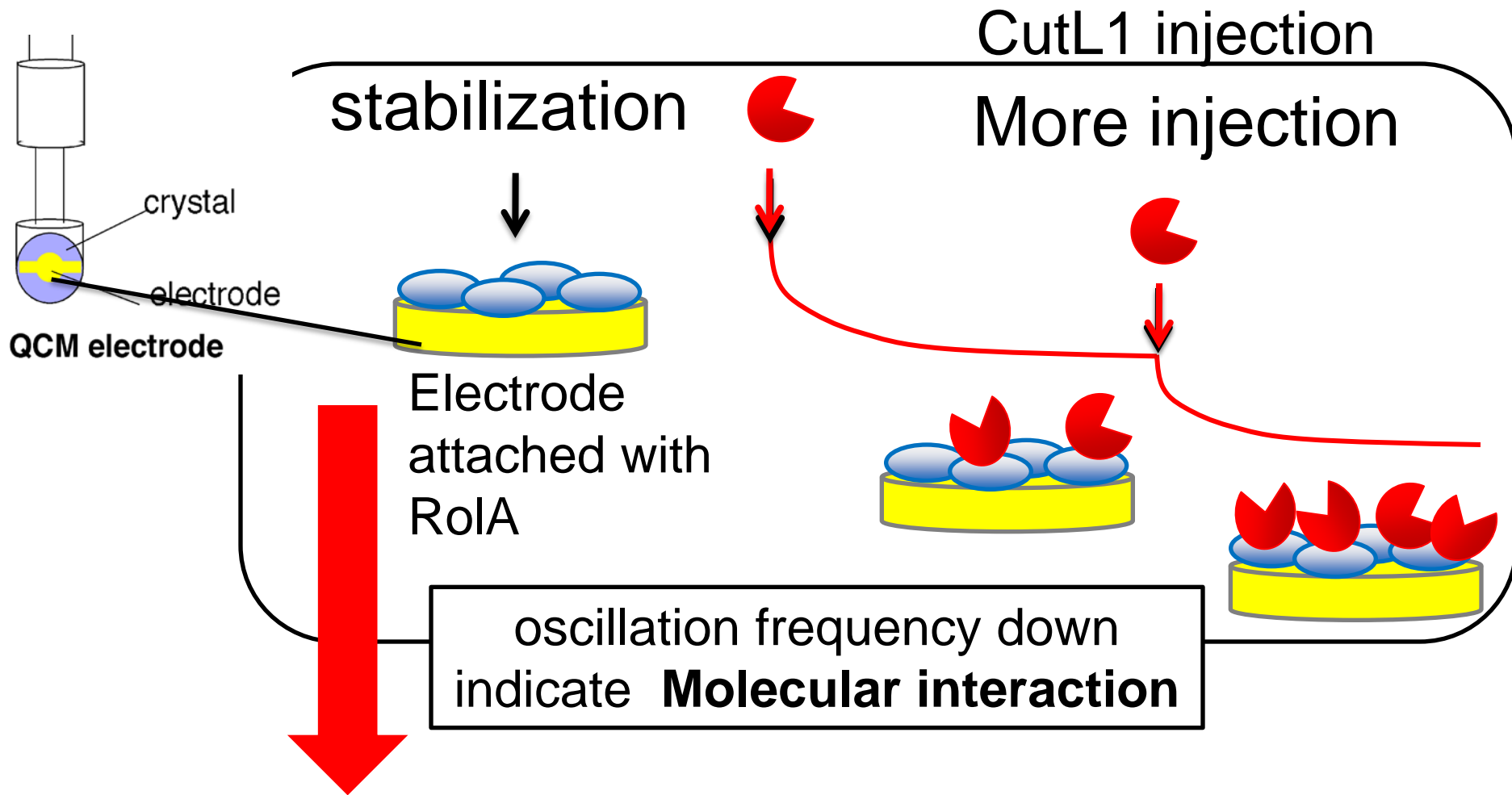


A quartz crystal microbalance (QCM) is a sensitive mass-measuring device. Its resonance frequency decreases linearly upon the increase of the mass on the QCM electrode at nanogram levels. Change of 100Hz of frequency corresponds to 3ng of protein bound to the QCM electrode.

## QCM electrode

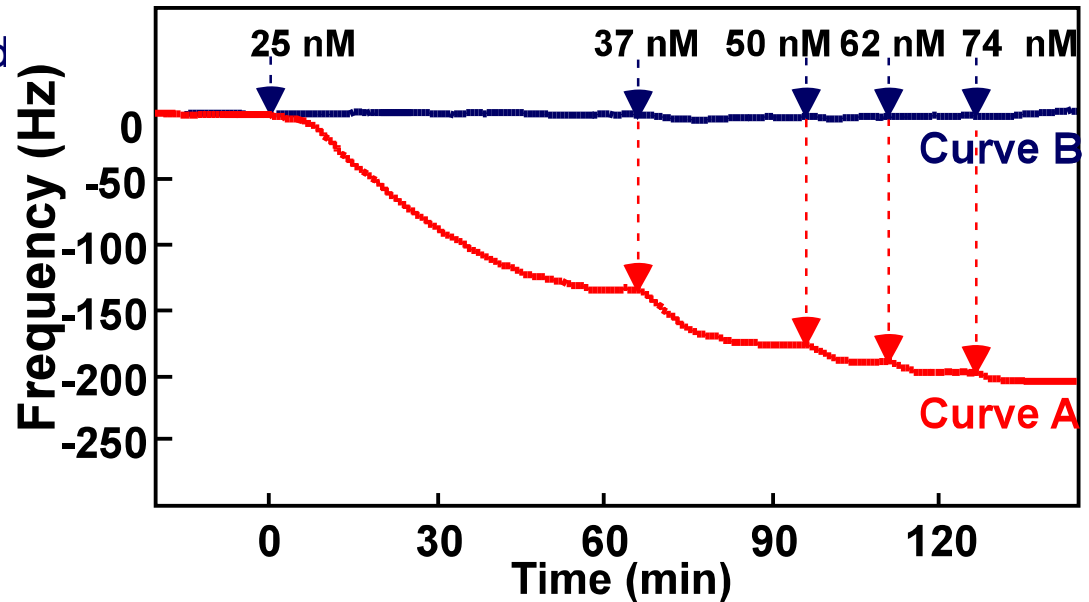
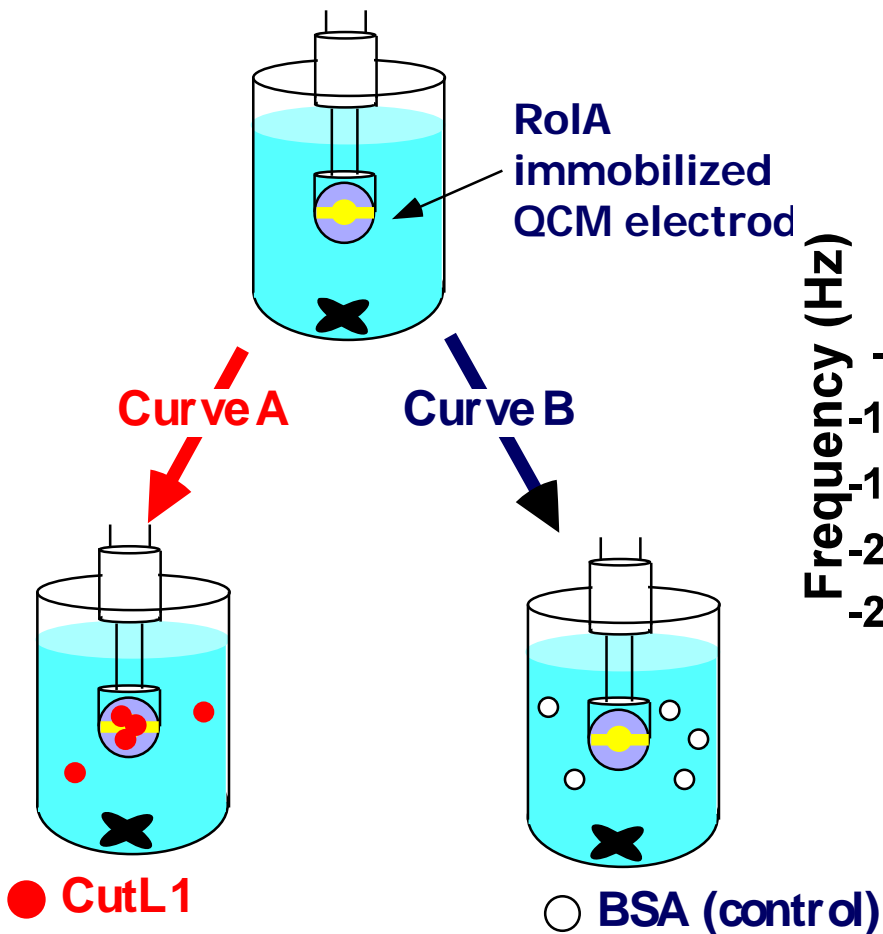


# About QCM



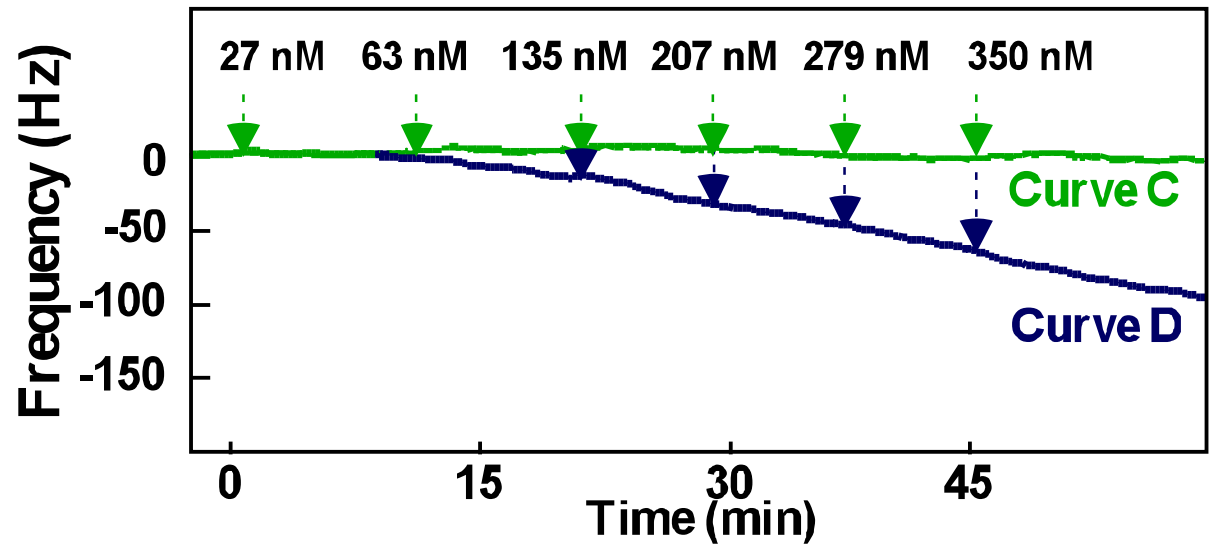
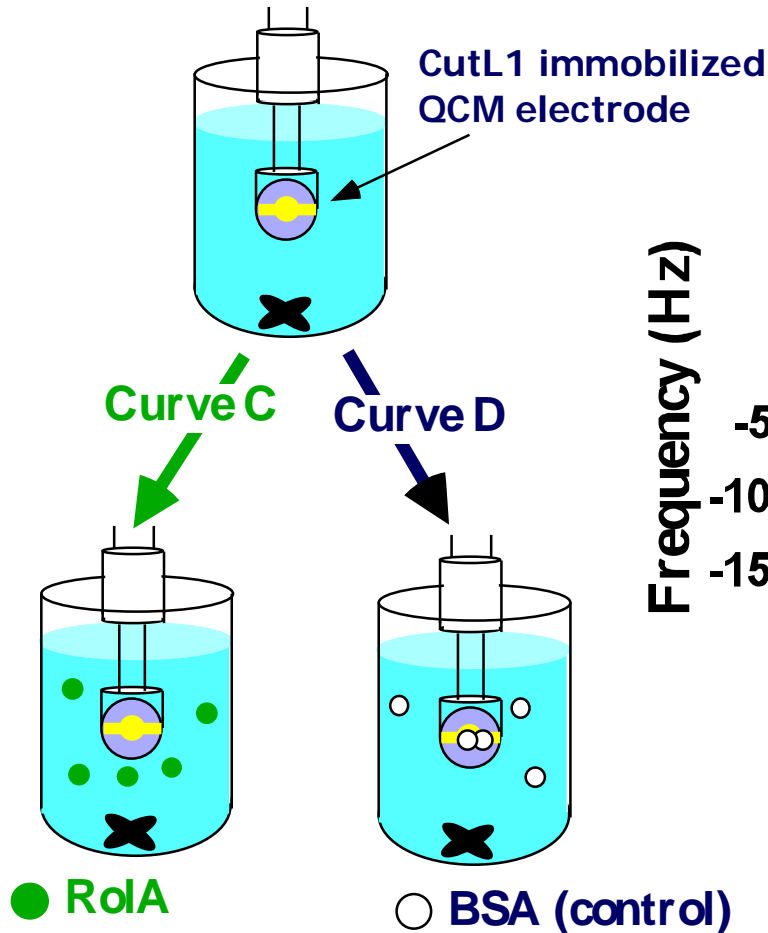
$K_D$  values which indicate binding affinity can be analyzed by monitoring oscillation frequency change.

# RoIA (ligand) – CutL1 (analyte) interaction



※ Concentrations in the figure indicate final concentrations of CutL1 and BSA in QCM step-wise analyses.

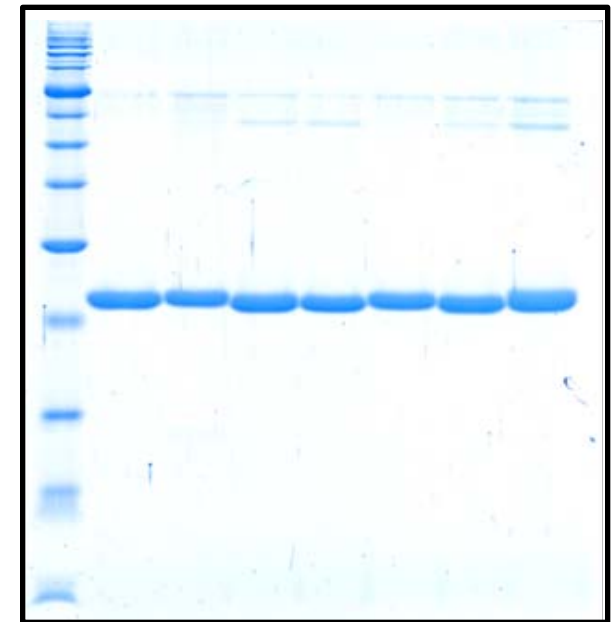
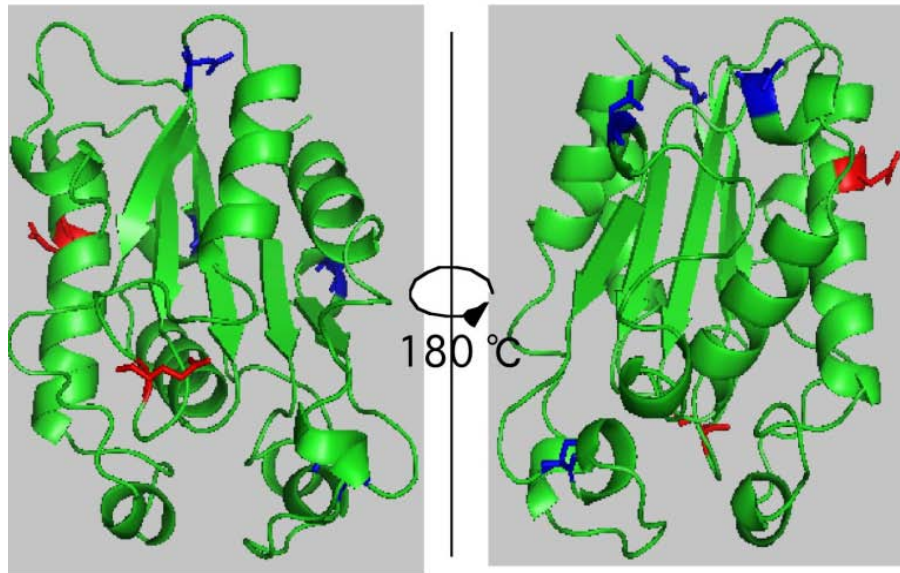
# CutL1 (ligand) – RoIA (analyte) interaction



※ Concentrations in the figure indicate final concentrations of RoIA and BSA in QCM step-wise analyses.

# Preparation of CutL1 mutants for RoIA-CutL1 binding assay

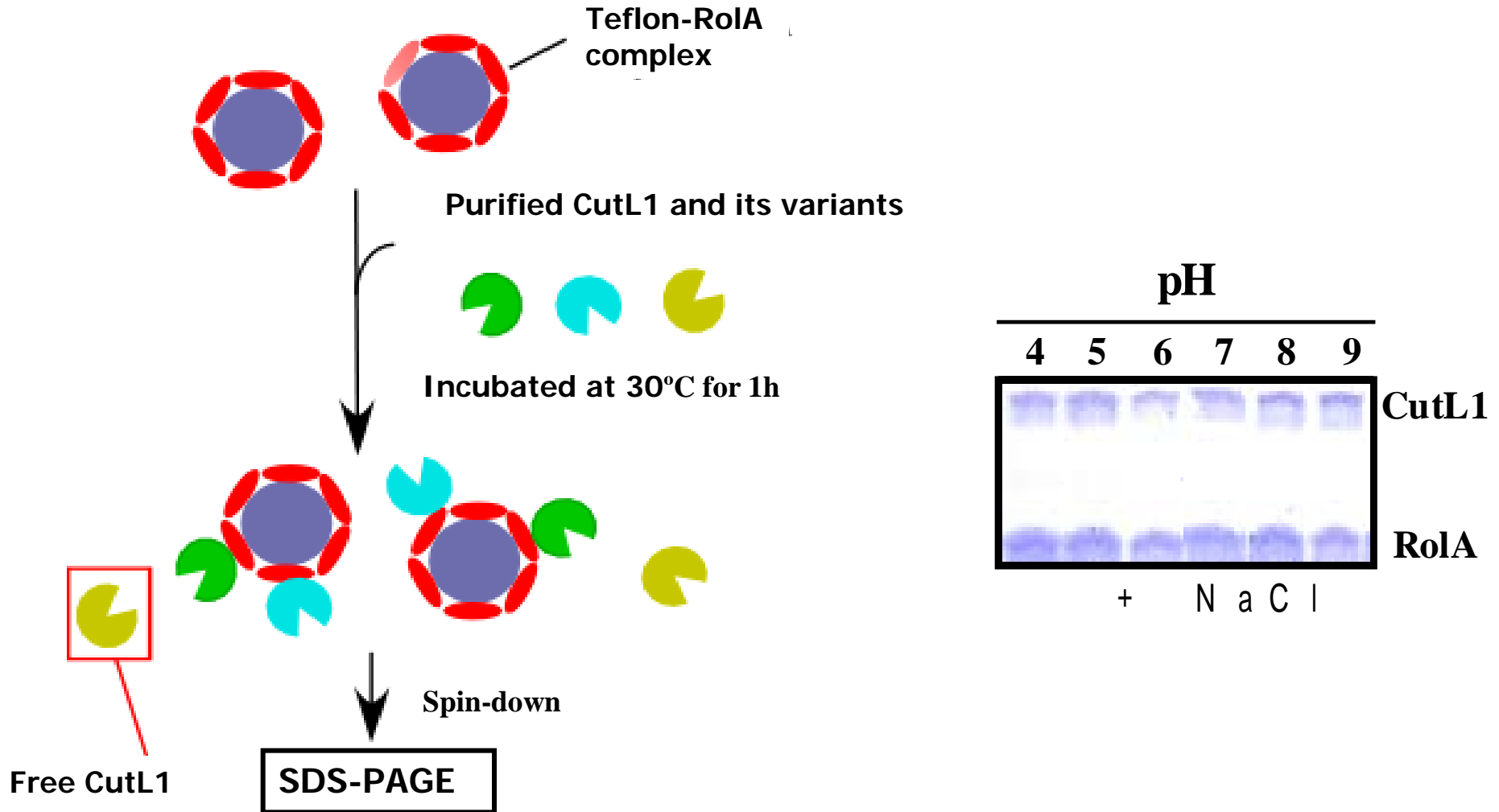
Six acidic amino acid residues (**Glu<sup>31</sup>**, **Glu<sup>109</sup>**, **Asp<sup>142</sup>**, **Asp<sup>145</sup>**, **Asp<sup>171</sup>**, **Asp<sup>203</sup>**) were substituted with Ser, and all variants produced by *A. oryzae* were purified. .



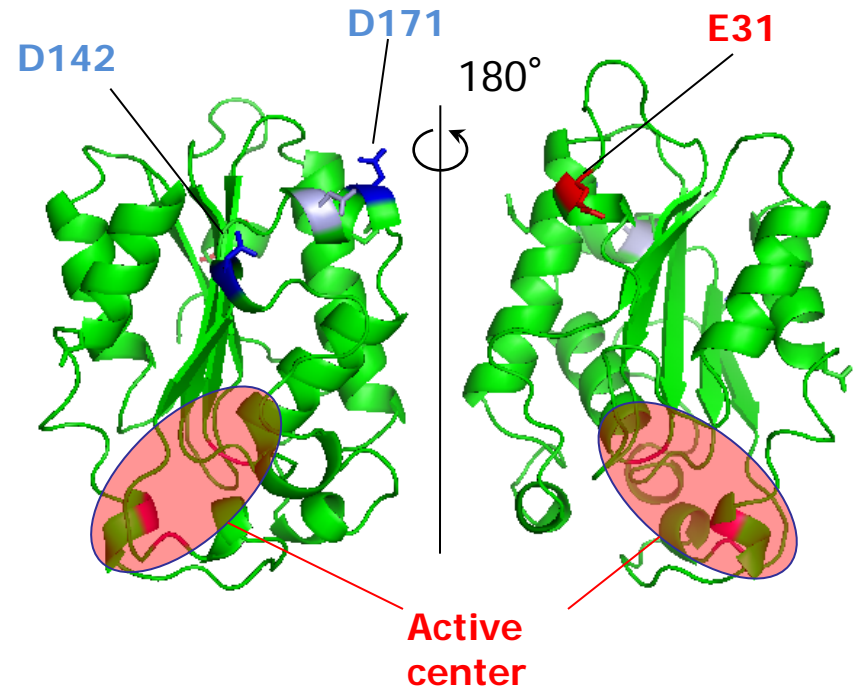
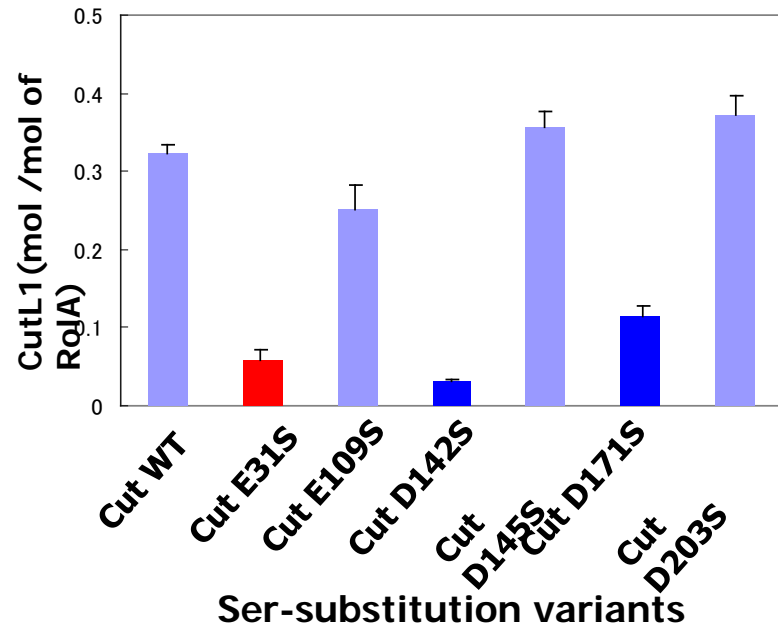
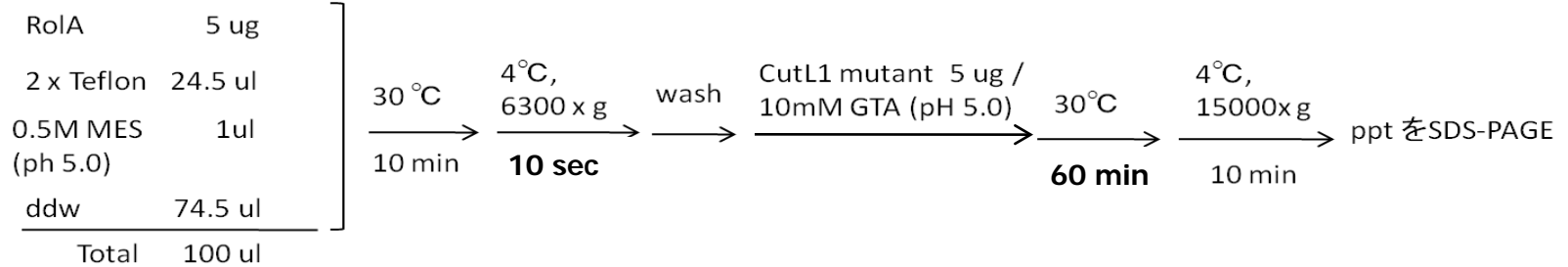
Cut WT  
Cut E31S  
Cut E109S  
Cut D142S  
Cut D145S  
Cut D171S  
Cut D203S

CutL1mutant の SDS-PAGE

# RoIA-CutL1 binding assay

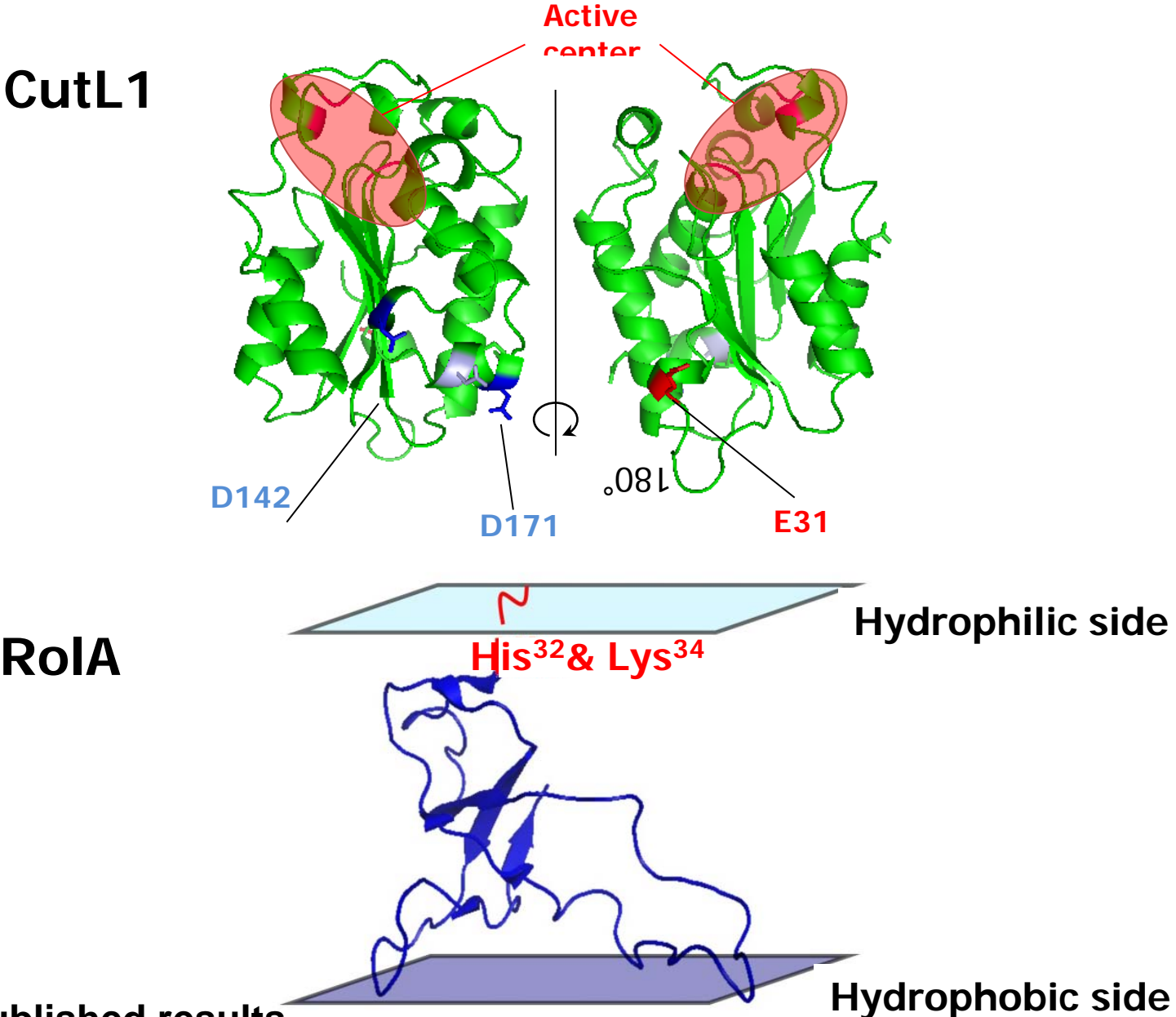


# Acidic amino acid residues (Asp, Glu) of CutL1 involved in CutL1-RoIA interaction



**E31, D142, D171** are important residues for CutL1-RoIA interaction.

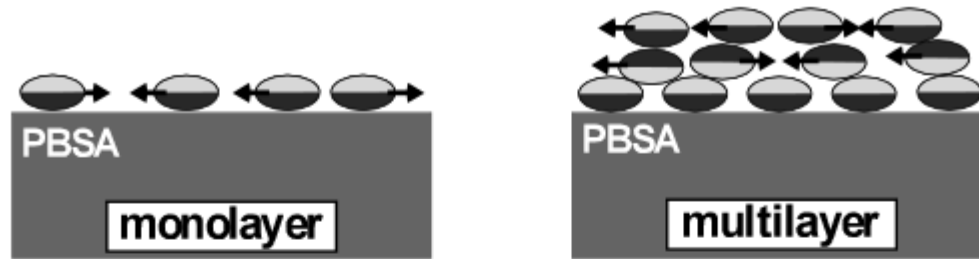
# Schematic model of RoIA-CutL1 interaction



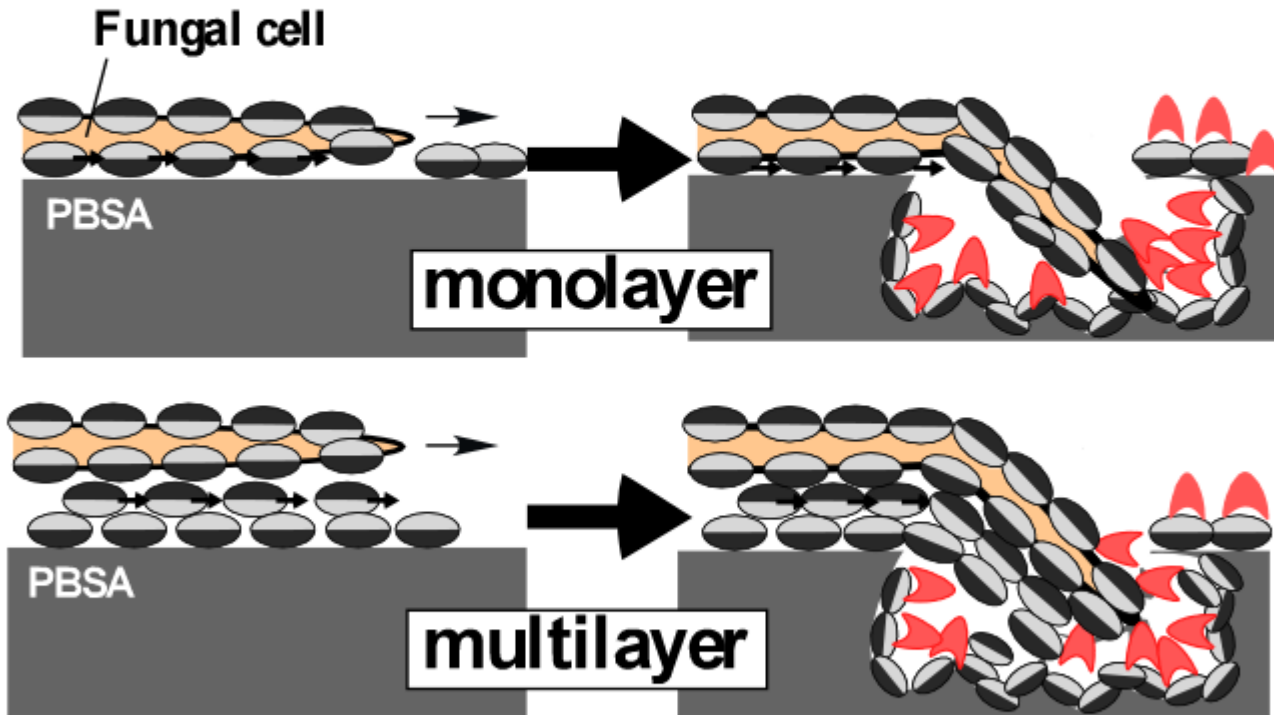
Abe et al. unpublished results

# Models of PBSA degradation by RoIA with CutL1

A



B



hydrophilic side



hydrophobic side



CutL1 molecule

# SUMMARY

- RoIA adsorbed to the PBSA surface recruits CutL1 to the surface, resulting in condensation of CutL1 and stimulation of PBSA hydrolysis.
- Glu31, Asp142 and Asp171 of CutL1 are critically required for the RoIA-CutL1 interaction by multivalent effect.
- H32 and K34 of RoIA are important for the RoIA-CutL1 interaction.

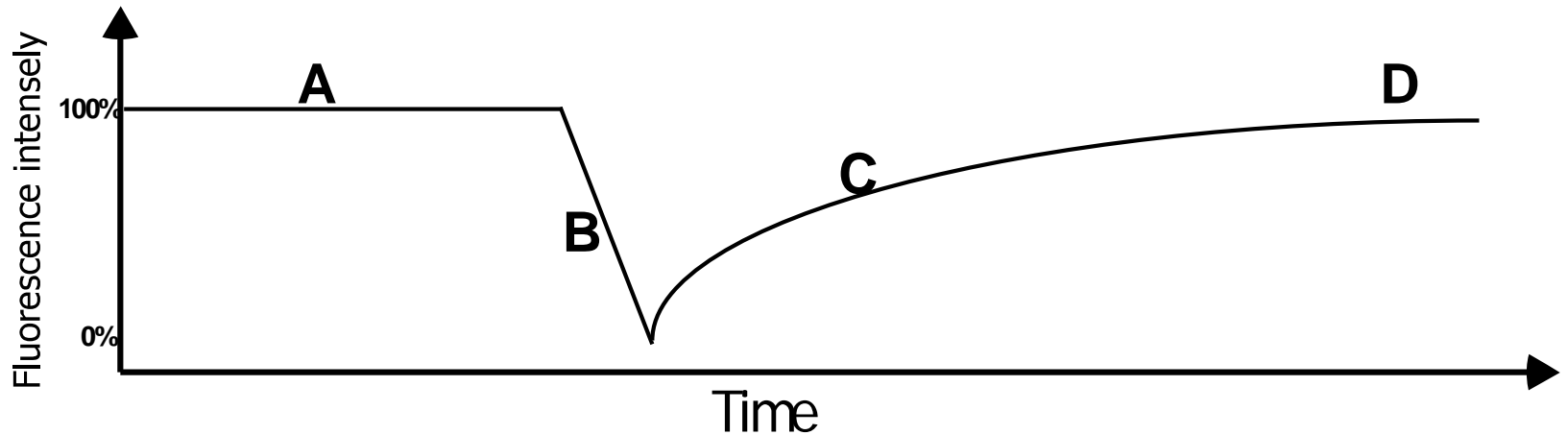
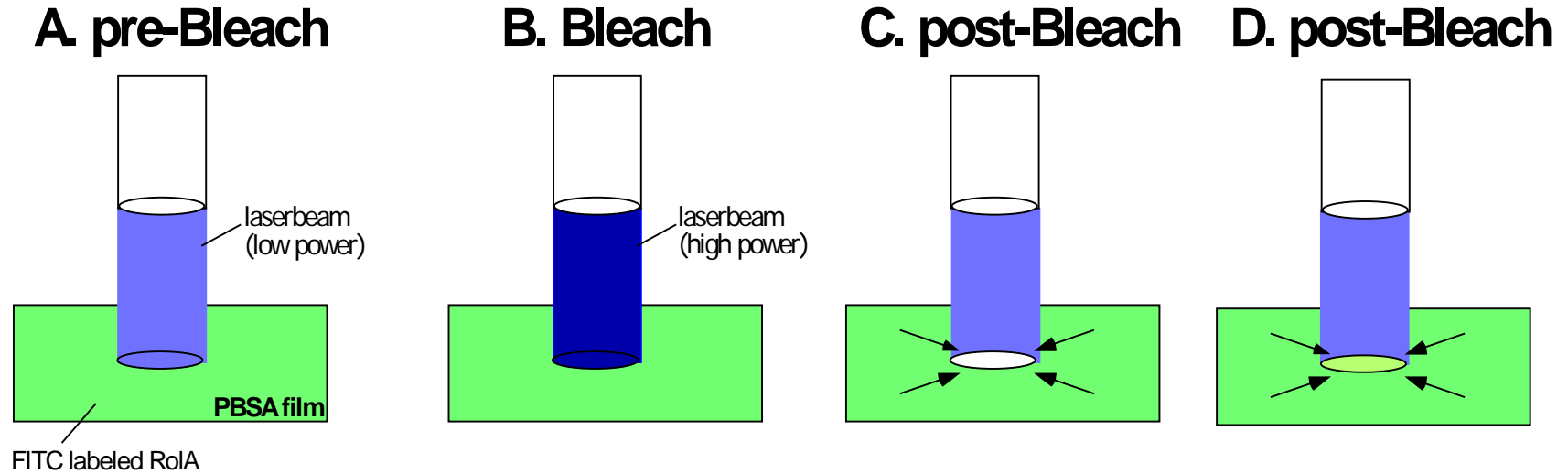
# ACKNOWLEDGEMENT

- RoIA  
Muragaki K., Uehara K., Ohataki S., Maeda H, Yamagata Y., Gomi K.,  
Hasegawa F. (Tohoku Univ.)
- *A. oryzae* genomics  
Machida M. and Asai K.( AIST)
- Recycle of PBSA  
Ishioka R. and Okino Y.(Showa High Polymer)
- CD analysis of RoIA  
Wang X. and Robillard G.T.(Groningen Univ.)
- FRAP analysis  
Kato M., Komura M. and Sato M. (Olympus Corp.)
- QCM analysis  
Jitsukawa T. (Inisium Corp.)
- AFM analysis  
Hondo H. (Ritsumeikan UNIV)

# REFERENCES

- Takahashi T., H. Maeda, S. Yoneda, S. Ohtaki, Y. Yamagata, F. Hasegawa, K. Gomi, T. Nakajima and **\*K Abe**, The fungal hydrophobin RoIA recruits polyesterase and laterally moves on hydrophobic surfaces, *Mol. Microbiol.*, **57**, 1780-1798 (2005)
- Ohtaki S., H. Maeda, T. Takahashi, Y. Yamagata, F. Hasegawa, K. Gomi, T. Nakajima and **\*K Abe**, Characterization of a novel hydrophobic surface binding protein HsbA produced by *Aspergillus oryzae* and its role in degradation of polybutylene succinate-co-adipate, *Appl. Environ. Microbiol.*, **72**, 2407-2413 (2006)
- **\*Abe K.**, K. Furukawa, T. Fujioka, D. Hagiwara, H. Maeda, J. Marui, O. Mizutani, T. Takahashi, A. Yoshimi, Y. Yamagata, K. Gomi, F. Hasegawa, “Novel Industrial Applications of *Aspergillus oryzae* Genomics.” *Aspergillus: Molecular Biology and Genomics*, Chapter 10, pp. 199-227, Machda M. and Gomi K. eds., Caister Academic Press (2010)

# Fluorescence recovery after photo-bleach (FRAP)



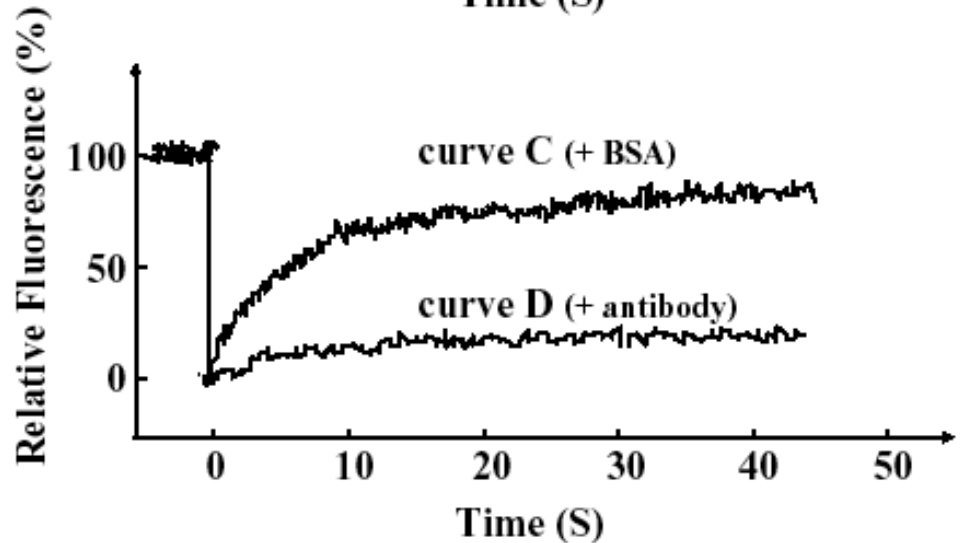
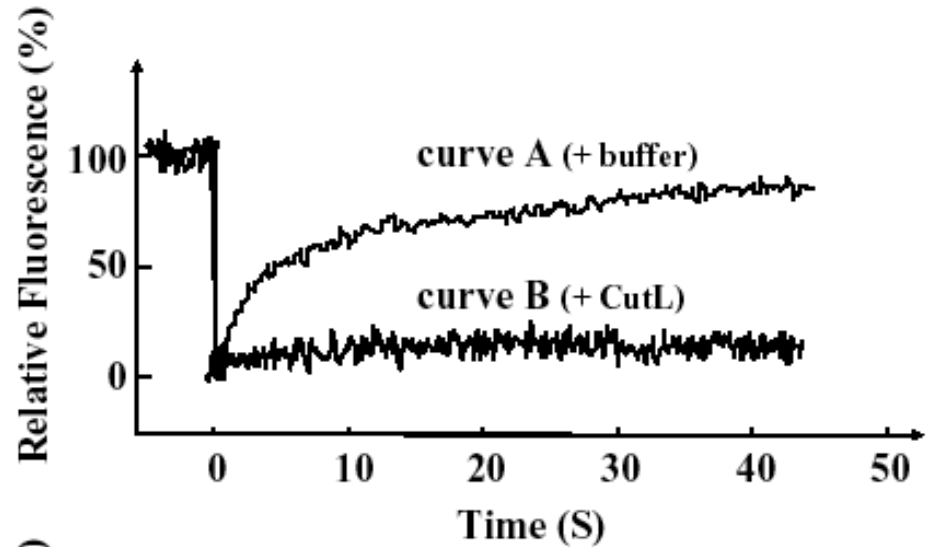
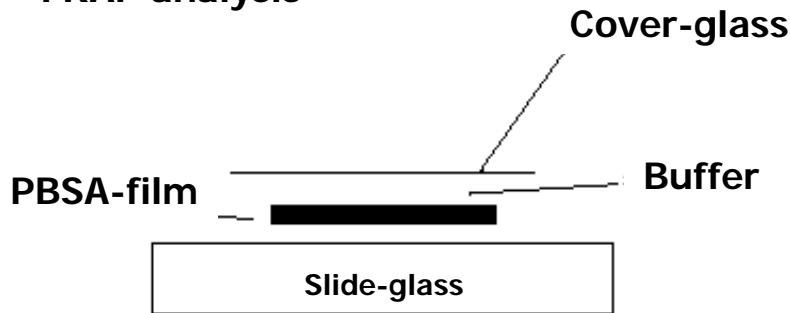
# FRAP analysis of FITC-RoIA adsorbed to PBSA films

FITC labeling of RoIA

↓  
Immobilization of FITC-RoIA on PBSA film

↓  
PBSA surface is overlaid with  
(A) buffer, (B) CutL1, (C) BSA, or  
(D) anti-RoIA antibody

↓  
FRAP analysis



- RoIA is laterally mobile on PBSA.
- Proteins that can interact with RoIA inhibits the mobility.

# A phylogenetic relationship between HsbA and 4MeS

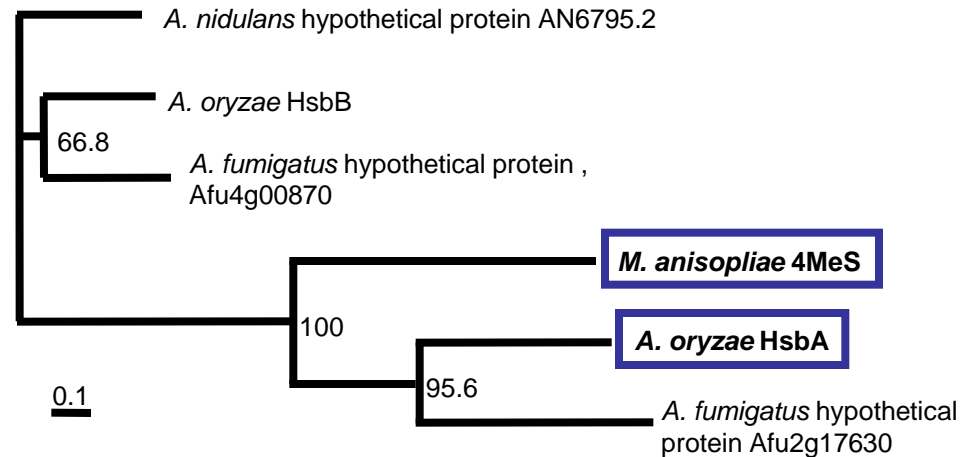
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1  ATGCTTGCCAAACACGTCCTTGCTGTCCTTCTGTCTGTTGGCGCTTCCGCCATTCTTTTC
6
1  M L A K H V L A V L L S V G A S A I P F 20
61  GACAAGCGAGATGCATCCGCTGTCCTCGCTGATTTTAAACACTCTCAACCGACCTGTCG
1  D K R N-terminal sequence 2
21  D K R D A S A V L L A D F N T L S T D L S 40
121 GCTCTCGGCTCTGCTATTTTCGAGCTTCGATGGAACGCTCAATGGTGCCTTGGGCGTCCAG
1  A L G S A I S S F D G T L N G A L G V Q 60
41
1  CAGAAGGAAGGCCAAGTAGAGACGGCGTTGAAGCAAACCGTCAGCGATGTGAAAGCGTCT
2  8
61  Q K E G Q V E T A L K Q T V S D V K A S 80
241 ACTGCGTTCAGTGTCTGCTGACAGCACAAAGCGTGACCAATGCTGTGACTGGTCTGGAGCCT
3  0
81  T A F S A A D S T S V T N A V T G L E P 100
301 AGCATTGTGAACGTTTCTCAACGATCTCGTTTTCCAAGAAATCCGGTTTTGACTCTGTTGGC
3  6
101 S I V N V L N D L V S K K S G F D S V G 120
361 GTTACCAGCATTGTCGATCGGATCTCAACTCCCTCCATGATCTTACTGGCCAGCTGTCA
4  2
121 V T S I V V S D L N S L H D L T G Q L S 140
421 ACTGAGCTCCAGTCGAAGGTCA XXXXXXXXXX TGACGCATCCACTATTTCTGATGAGGCTGCG
4  0
141 T E L Q S K V T S G D A S T I S D E A A 160
441 CGA XXXXXXXXXX AAGGCATCGCTGGGTTATTCCTAG 525
161 R K K A I A A Y S * 175
    
```

## Hydrophobic Surface Binding protein A (HsbA)

### 4MeS

- Produced by an entomopathogenic fungus
- Transcribed in the fungal cells growing on the insect cuticle
- Function unknown

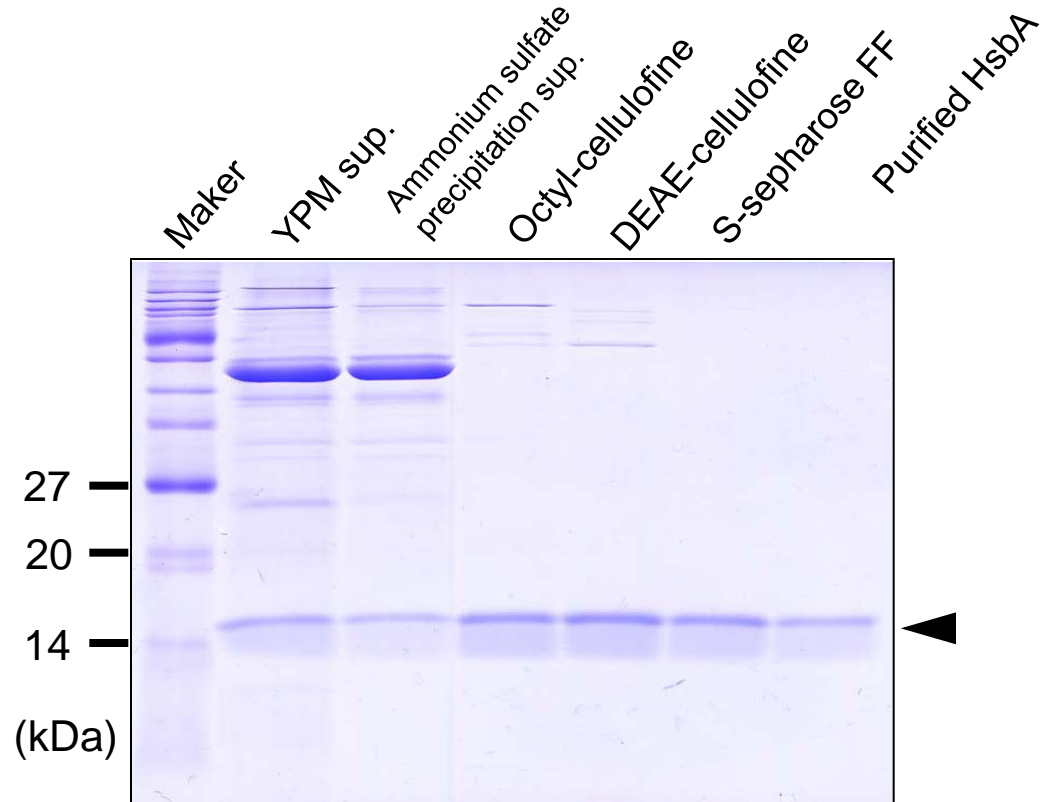
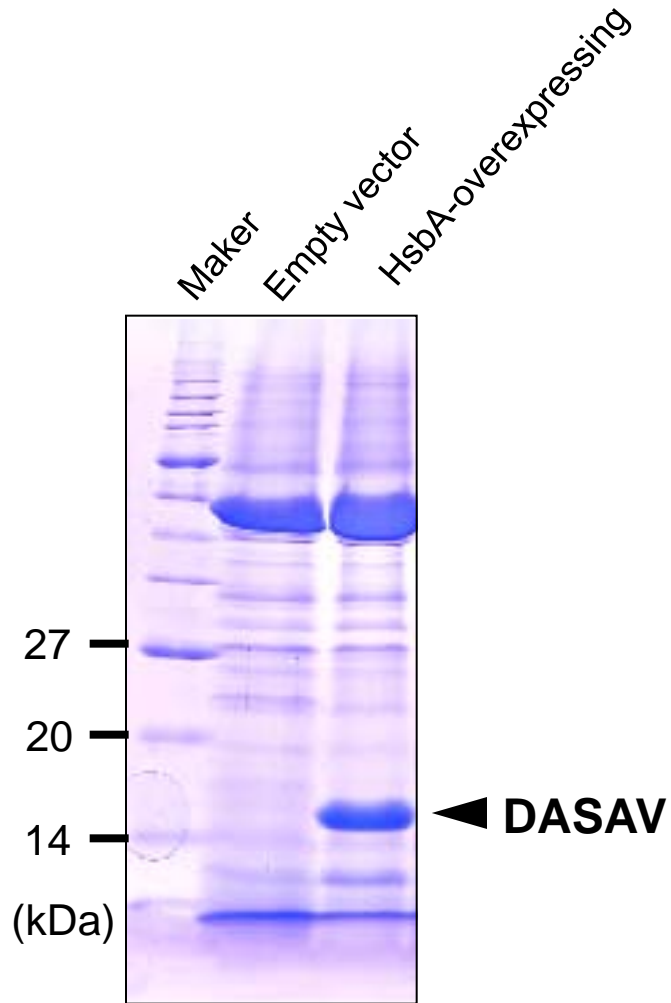


The phylogenetic tree of HsbA and its orthologues

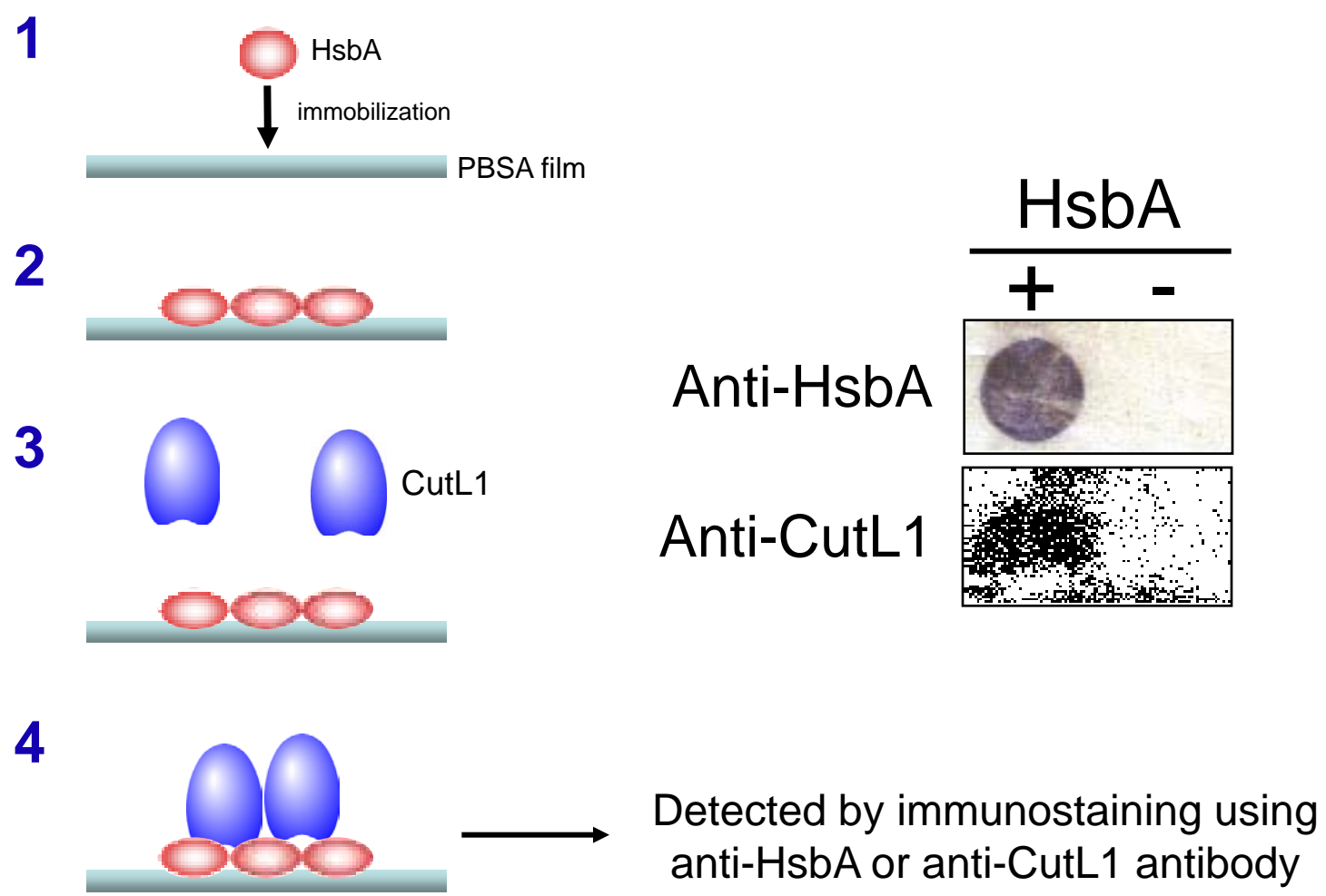


*M. anisopliae* growing on the surface of an insect

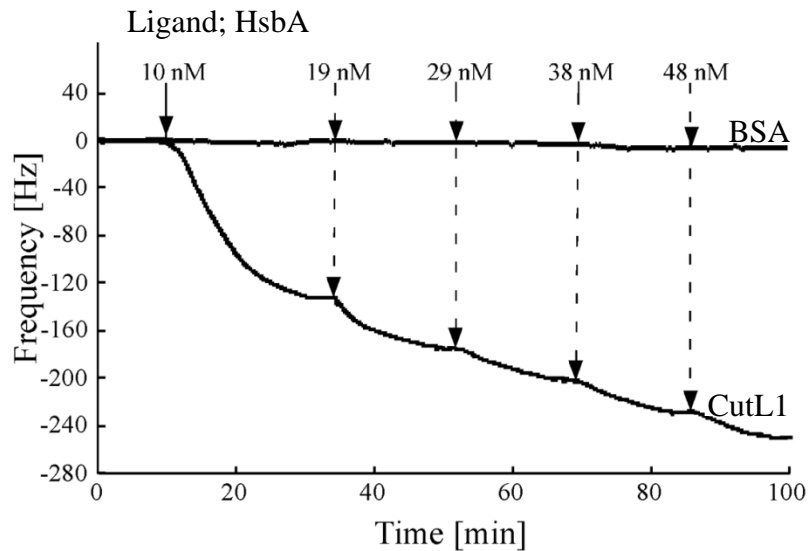
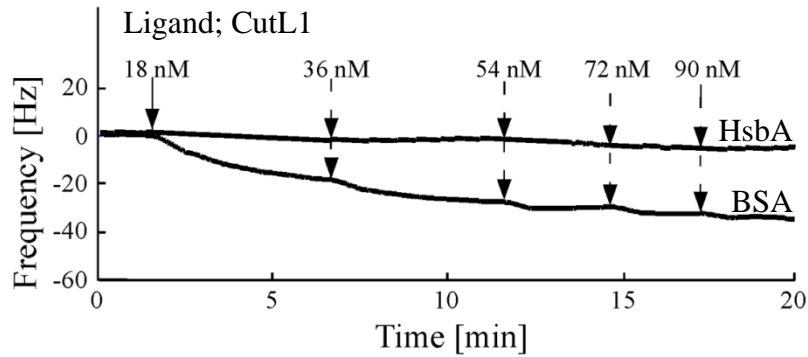
# Expression and purification of recombinant HsbA



# Recruitment of CutL1 on HsbA adsorbed on the PBSA surface



# Interaction between CutL1 and HsbA



**Interaction between soluble CutL1 and HsbA adsorbed on the hydrophobic surface by QCM analysis**

