

第6回(平成26年度第3回)CRCフォーラム(平成26年12月15日(月)開催)

「TDUで展開する先端微生物研究」

微生物酵素を用いた有用物質生産 —新規酵素活性を利用したペプチド合成法の開発—

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微生物酵素を用いた有用物質生産

—新規酵素活性を利用したペプチド合成法の開発—

安部智子

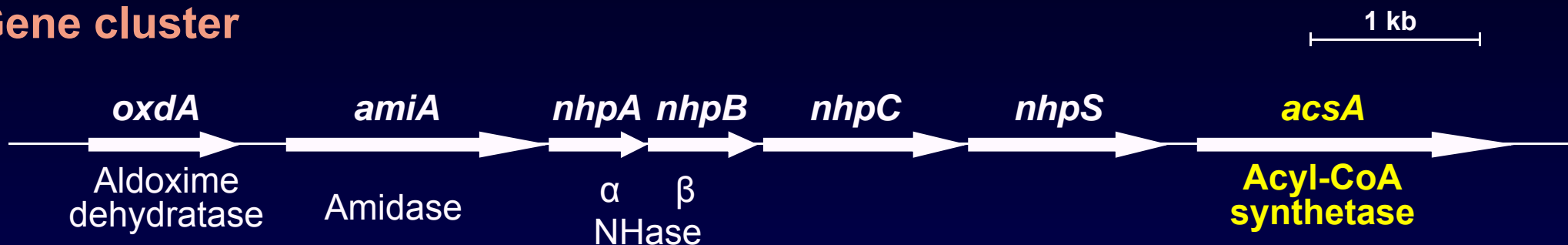
(東京電機大学 理工学部 生命理工学系・助教)

2014/12/15

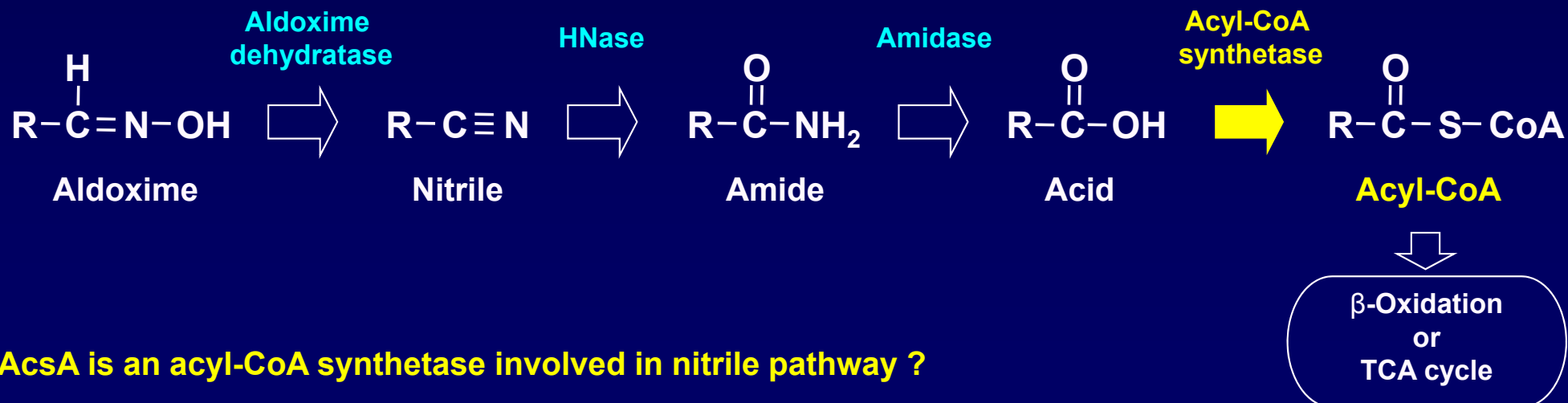
ニトリル代謝経路に関与するアシルCoA合成酵素の発見

Pseudomonas chlororaphis B23

Gene cluster

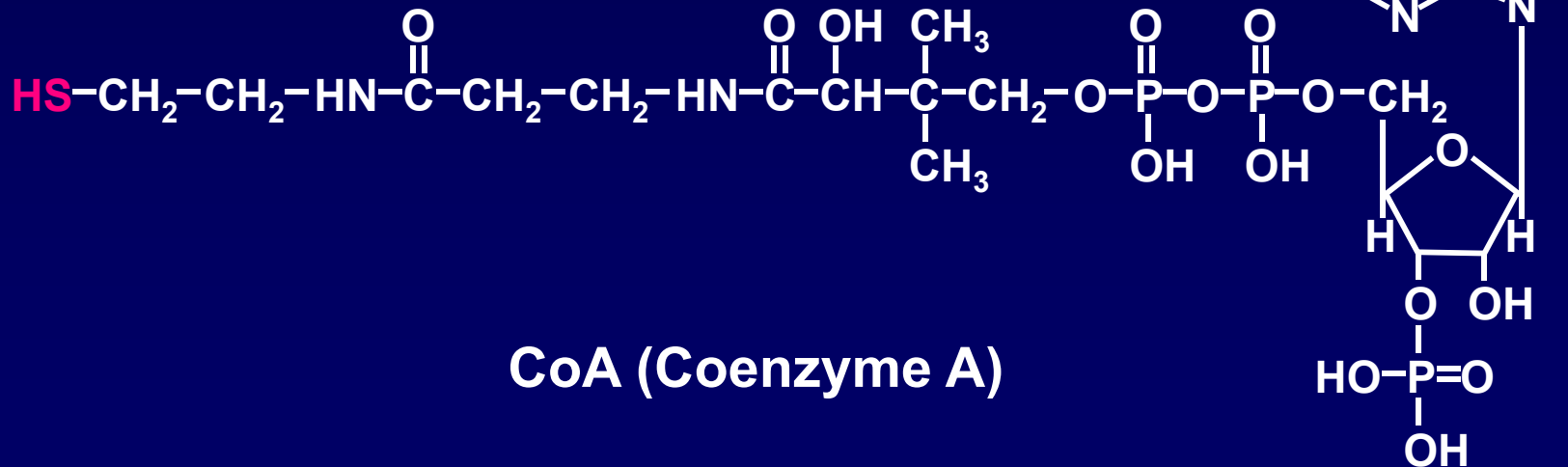
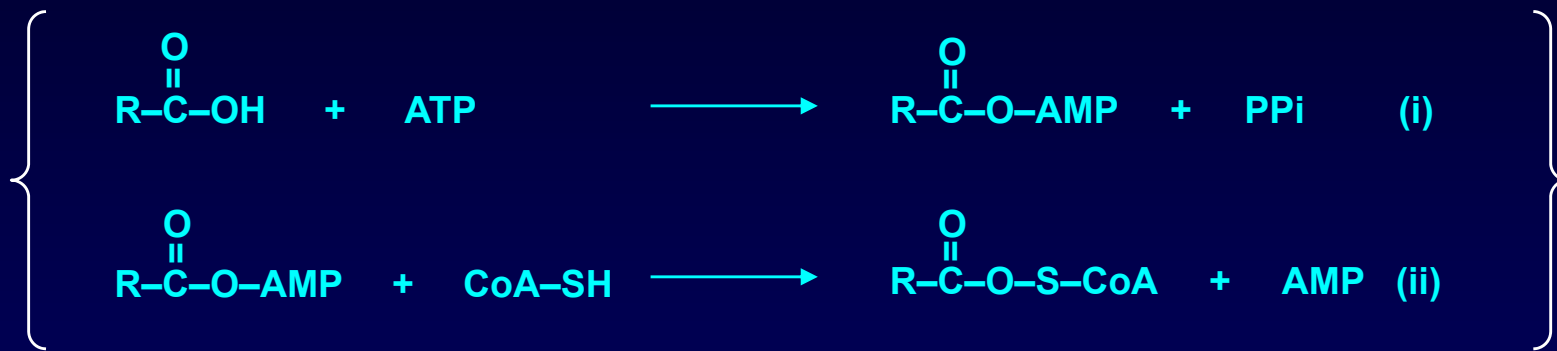
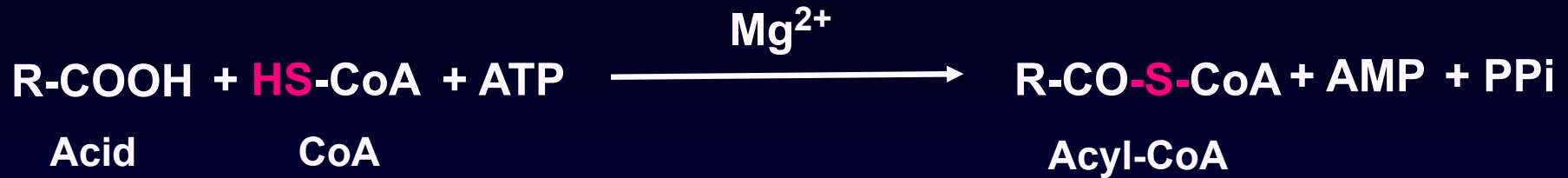


Pathway



AcsA is an acyl-CoA synthetase involved in nitrile pathway ?

アシルCoA合成酵素によるアシルCoA合成反応



アシルCoA合成酵素

Acetyl-CoA synthetase

Supply of acetyl-CoA for biosynthesis of fatty acid and cholesterol

Acyl-CoA synthetase

Short-chain acyl-CoA synthetase
(C2-C5)

Activation of fatty acid (C2-C5) for β -oxidation

Medium-chain acyl-CoA synthetase
(C4-C12)

Activation of fatty acid (C4-C12) for β -oxidation

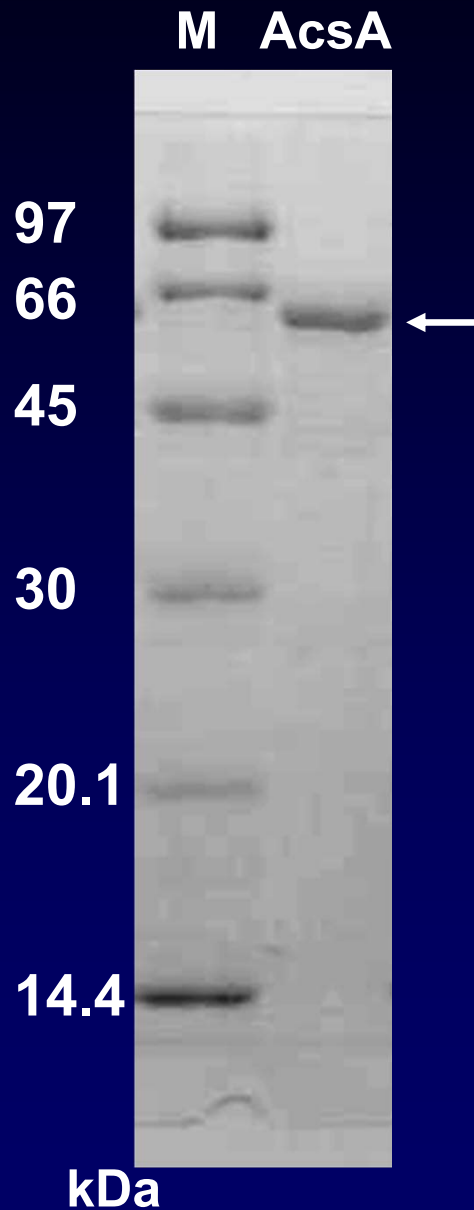
Long-chain acyl-CoA synthetase
(C10-C22)

- Activation of fatty acid (C10-C22) for lipid (triglyceride, phosphatide, and cholesterol ester) synthesis
- Activation of fatty acid for β -oxidation

Very long-chain acyl-CoA synthetase
(C24-)

- Activation of fatty acid (C24-) for β -oxidation
- Fatty acid transport protein (FATP)

AcsA の基質特異性 (脂肪酸)

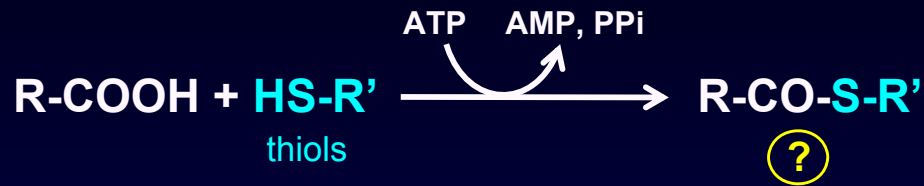


Substrate	Relative k_{cat}/K_m^* (%)	
Formic acid (C1)	0.00105	Aliphatic acids
Acetic acid (C2)	0.166	
Propionic acid (C3)	7.59	
Butyric acid (C4)	13.6	
Valeric acid (C5)	23.8	
Hexanoic acid (C6)	0.558	
Heptanoic acid (C7)	0.00345	
Octanoic acid (C8)	N.D.**	
Isobutyric acid	100	
dl-Isovaleric acid	0.612	
dl-2-Hydroxybutyric acid	0.400	Aromatic acids
dl-3-Hydroxybutyric acid	0.0284	
Acrylic acid	1.56	
trans-Crotonic acid	2.94	
Benzoic acid	0.222	
3-Phenylpropionic acid	0.00379	
4-Phenylbutyric acid	N.D.	
Picolinic acid	0.00128	
Nicotinic acid	N.D.	
Isonicotinic acid	0.00371	

* k_{cat}/K_m = Catalytic efficiency **N.D = Not detected

The substrate specificity of AcsA is similar to those of aldoxime dehydratase, NHase, amidase in *P. chlororaphis* B23.

アシルCoA合成酵素 (AcsA) における新規酵素反応の発見



Reaction mixture

200 mM Tris-HCl (pH 7.5)
 8 mM MgCl₂
 100 mM Ammonium sulfate
 5 mM ATP
 10 mM Isobutyrate
 10 mM Thiols
 Purified enzyme (AcsA)

Thiol compounds

Cysteamine (2-Mercaptoethylamine)

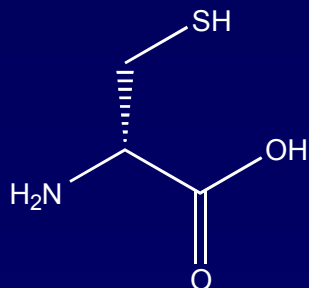


Glutathione (reduced form)

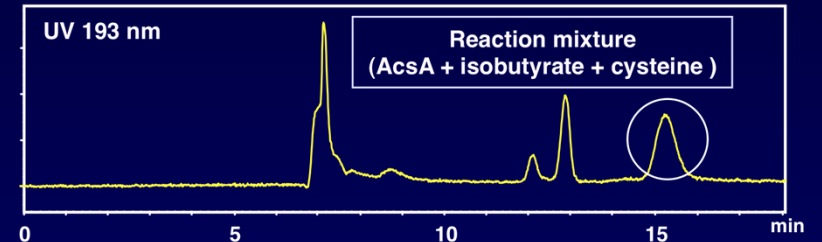


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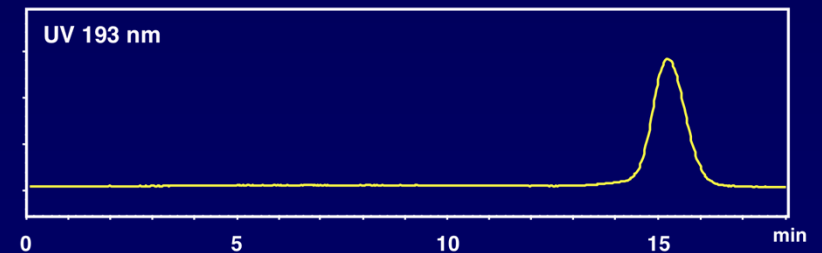
L-Cysteine



Increase in AMP



Purification



HPLC analysis

Column Shim-Pack SCR-102H
 Mobile phase 0.1 % formate (pH 3.0)
 Column temp. 40°C
 Detection 193 nm

予測される新規反応産物

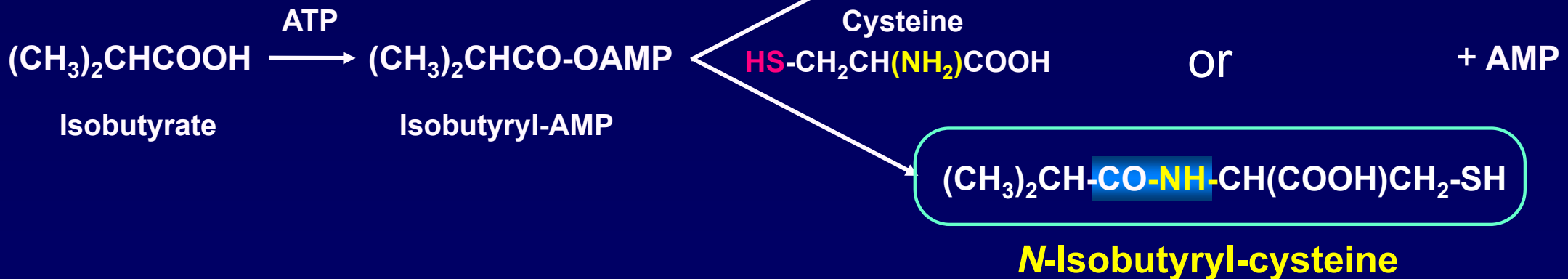
High-resolution FAB-MS (Fast Atom Bombardment Mass Spectrometry)

Mass spectrometer JMS-HX110A
Matrix Glycerol

Observed m/z
192.0713

Composition $C_7H_{14}NO_3S$

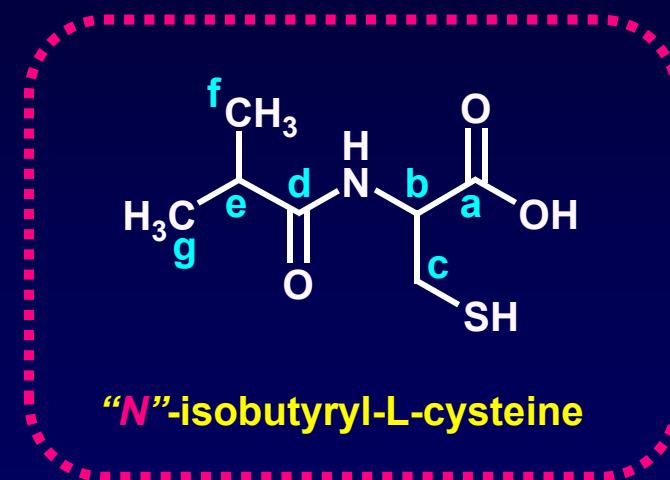
Predicted reaction



反応産物の同定

NMR spectral analysis

	δ_C	δ_H
Purified product		
a	176.234	—
b	55.313	4.31415 (<i>dd</i> , 4.46, 6.170)
c	26.348	2.85385 (<i>dd</i> , 4.446, 14.153), 2.80415 (6.170, 14.018)
d	181.123	
e	35.266	2.4656 (<i>q</i> or <i>qq</i>)
f	19.185	0.96895 (<i>d</i> , 5.739)
g	18.720	
Authentic <i>N</i>-isobutyryl-L-cysteine		
a	174.182	—
b	55.034	4.4292 (<i>dd</i> , 4.683, 6.993)
c	25.410	2.85385 (<i>dd</i> , 4.683, 14.205), 2.80415 (6.993, 14.178)
d	181.650	
e	35.062	2.4656 (<i>q</i> or <i>qq</i>)
f	19.024	0.96895 (<i>d</i> , 6.913)
g	18.765	

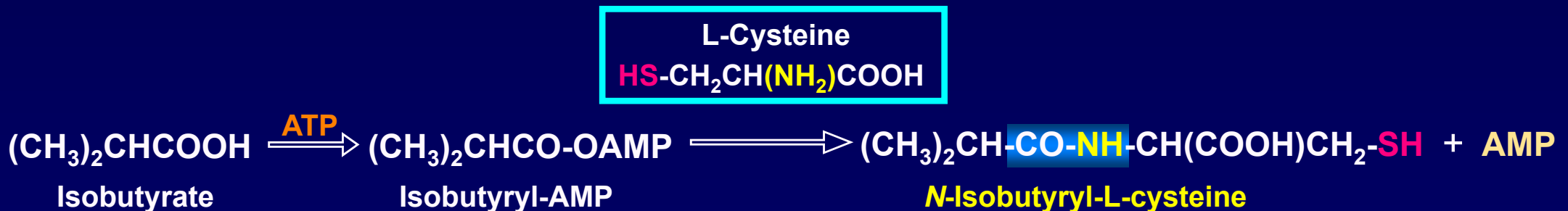


AcsA による *N*-アシル化反応 (amide bond formation)

Original reaction



Novel reaction



N-アシル化反応における基質特異性 (脂肪酸基質)

Reaction mixture

200 mM Tris-HCl (pH 7.5)
5 mM ATP
8 mM MgCl₂
100 mM Ammonium sulfate
10 mM Acid (short-chain)
10 mM L-Cysteine
Purified enzyme (AcsA)



AMP detection



Product separation



LC-ESI-MS

{
N-Propionyl-L-cysteine
N-Butyryl-L-cysteine
N-acrylyl-L-cysteine

Acid substrate	Relative activity (%) [*]
Acetate	74
Propionate	108
Butyrate	89
Isobutyrate	100
Valerate	90
Hexanoate	46
Heptanoate	6
Acrylate	138
Crotonate	94

^{*}The reactions were carried out at 20°C except that various acids were used as the substrates in place of isobutyrate, corresponding to 0.175 μmol AMP/min/mg protein (100%).

Superfamily of adenylate (AMP)-forming enzymes

Acetyl-CoA synthetase

Acyl-CoA synthetase (short-, medium-, long-, very long-)

AcsA: Short-chain acyl-CoA synthetase from *P. chlororaphis* B23

AcesEco: Acetyl-CoA synthetase from *E. coli*

Aces1Scer: Acetyl-CoA synthetase from *S. cerevisiae*

FadK: Middle-chain acyl-CoA synthetase from *E. coli*

FadD: Long-chain acyl-CoA synthetase from *E. coli*

Long-ACSr: Long-chain acyl-CoA synthetase from rat liver

Firefly luciferase

: oxidation of luciferin to generate oxyluciferin and light (bioluminescence reaction)

LucPpy: Firefly luciferase from *Photinus pyralis*

LucLcr: Firefly Luciferase from *Luciola cruciata*

4-Coumarate:CoA ligase

: a key role in phenylpropanoid metabolism, providing precursors for a large variety of important plant secondary metabolites, such as lignin, flavonoids, and phytoalexins

4CL: 4-Coumarate:CoA ligase from Parsley

CCL: Cinnamate:CoA ligase from *S. coelicolor* A3(2)

Adenylation domain of nonribosomal peptide synthetase (NRPS)

PheA: phenylalanine-activating subunit of gramicidin synthetase 1 from *Bacillus brevis*

EntE: 2,3-DHB-activating subunit in enterobactin biosynthesis of *Escherichia coli*

DhbF1A, 2A, DhbE: adenylation domains of NRPS for bacillibactin biosynthesis in *Bacillus subtilis*

ACV1, 2, 3: adenylation domains of NRPS for penicillin biosynthesis in *Penicillium chrysogenum*

DltA: D-alanine:D-alanyl carrier protein ligase responsible for the initial step of lipoteichoic acid D-alanylation in Grampositive bacteria

N-acetyl-L-cysteine synthesis by acetyl-CoA synthetase

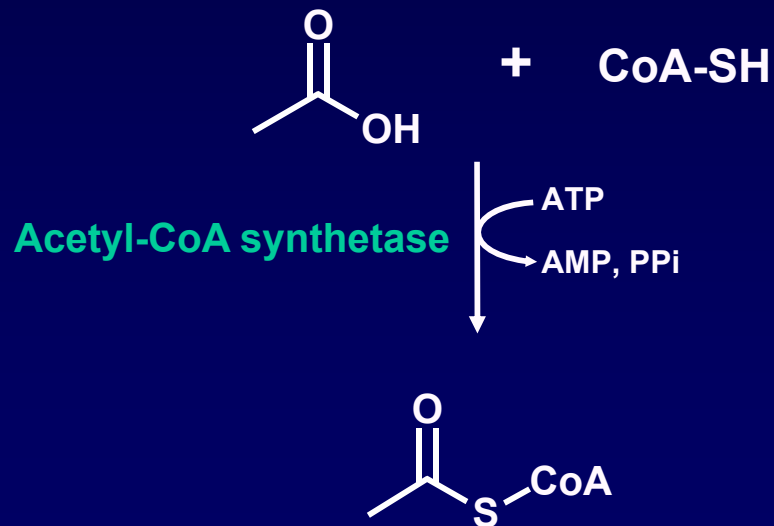
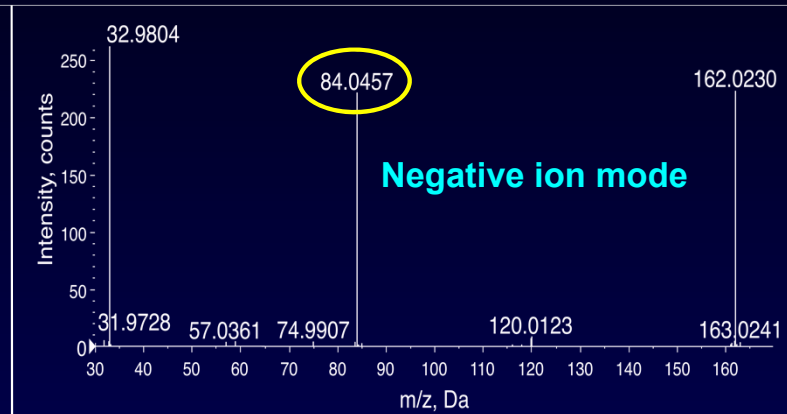
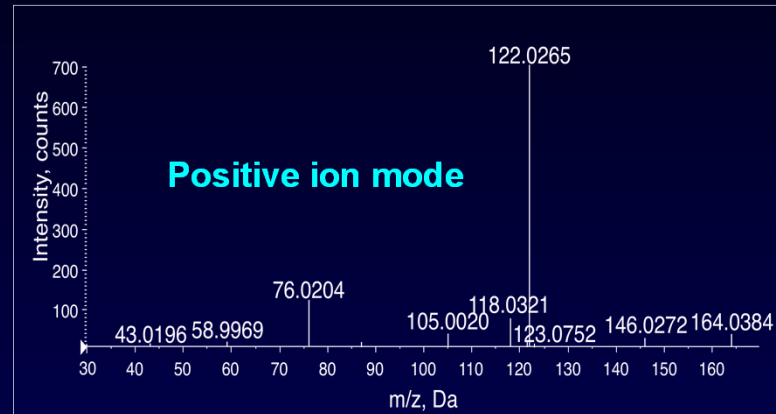
Reaction mixture
 200 mM Tris-HCl (pH 7.5)
 5 mM ATP
 8 mM MgCl₂
 100 mM Ammonium sulfate
 10 mM Acetate
 10 mM L-Cysteine
 + Purified enzyme

⇒ AMP detection

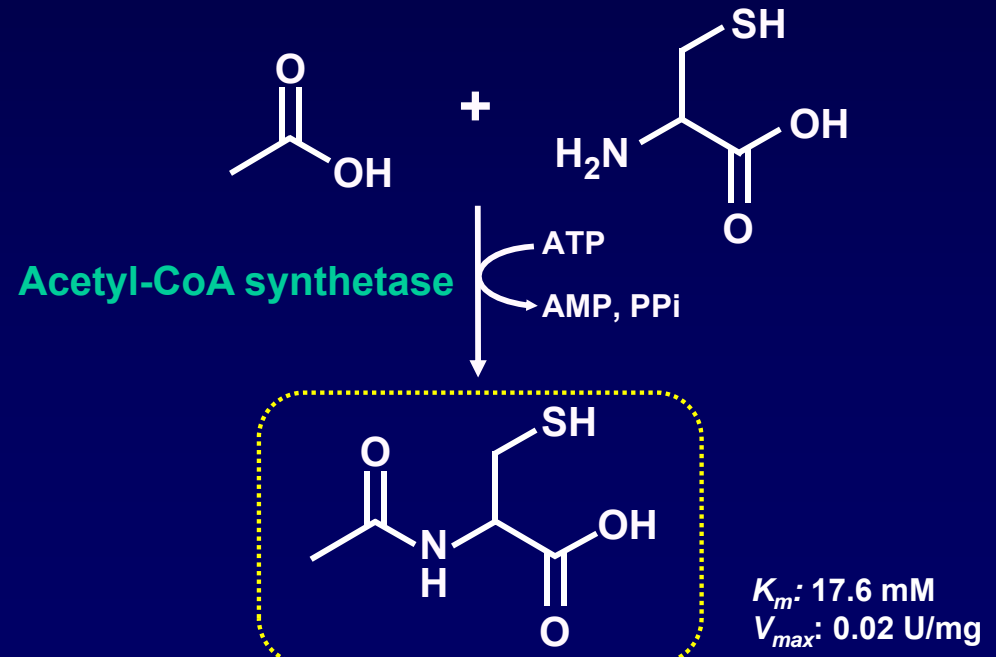
⇒ Product separation

⇒ LC-ESI-MS ($M_r = 163$)
MS/MS

Acetyl-CoA synthetase
from *Saccharomyces cerevisiae*



Acetyl-CoA synthesis (original)



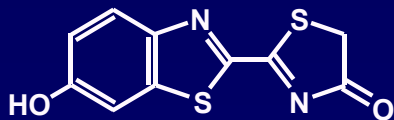
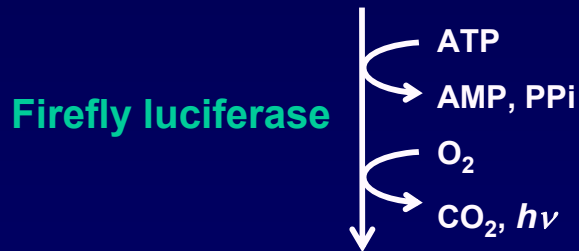
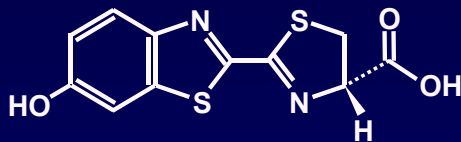
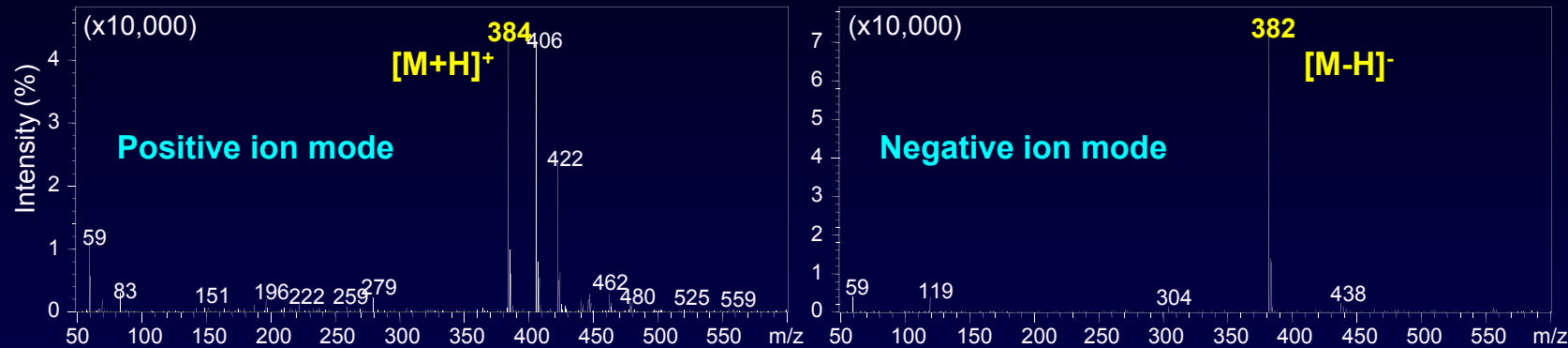
N-Acetyl-L-cysteine synthesis (new)

N-Luciferyl-L-cysteine synthesis by Firefly luciferase

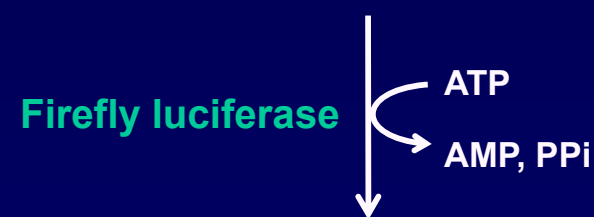
Reaction mixture
 200 mM Tris-HCl (pH 7.8)
 10 mM ATP
 16 mM MgCl₂
 10 mM D-Luciferin
 100 mM L-Cysteine
 + Purified enzyme

Firefly luciferase
 (*Photinus pyralis*)

⇒ AMP detection ⇒ Product separation ⇒ LC-ESI-MS ($M_r = 383$)



Bioluminescence reaction (original)



N-Luciferyl-L-cysteine synthesis (new)