



Title	海藻由来ハロゲン化酵素の特性
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北海道海洋科学シンポジウム

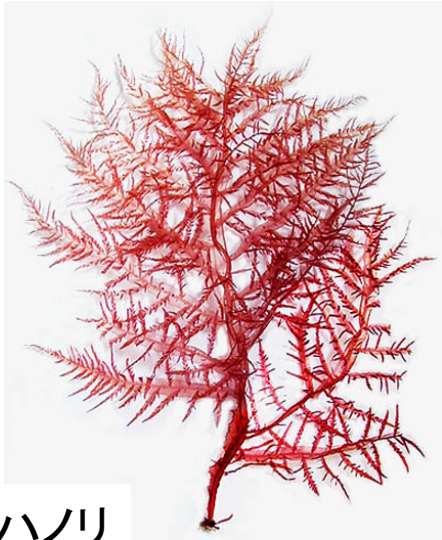
海藻由来ハロゲン化酵素の特性

2010年11月5日

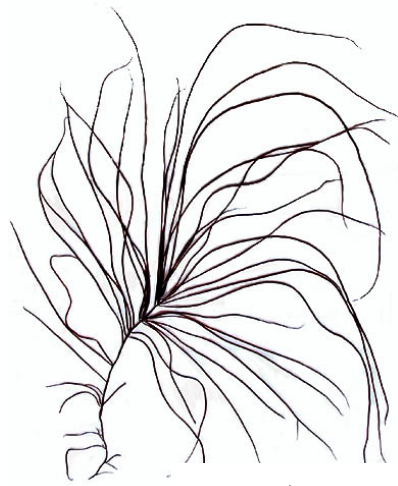
鳥取大学大学院・工学研究科・化学・生物応用工学専攻
大城 隆



鳥取県ホームページより



ヌネハノリ



ツルシラモ



トロロコンブ

日本では古くから多くの海藻を食料資源として利用

近年、海藻の持つ機能性に注目

- 有効成分の単離
- 生理機能解析

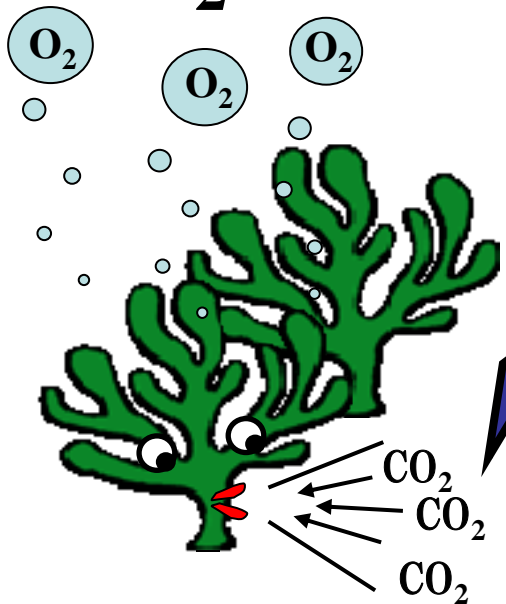
海藻の主な有効成分

高分子: アルギン酸、フコイダンなどの多糖類
低分子: 遊離アミノ酸、抗酸化成分など
無機物質: 微量元素

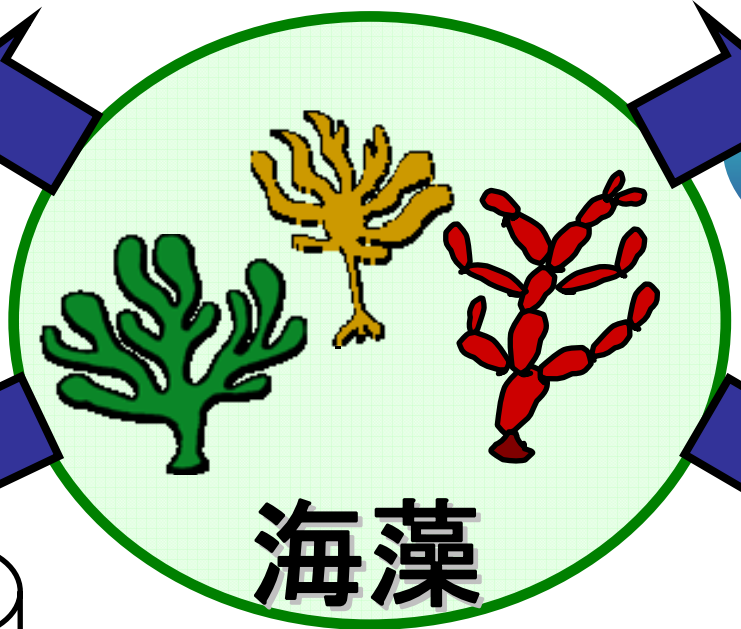
医薬品、化粧品、飼料、工業原料



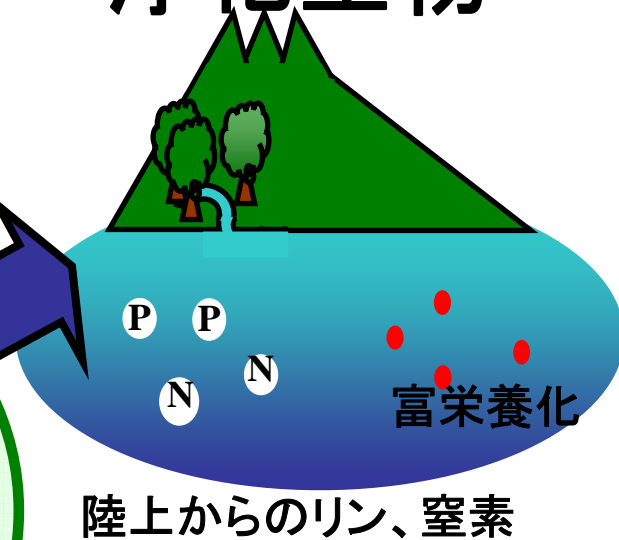
CO₂の削減



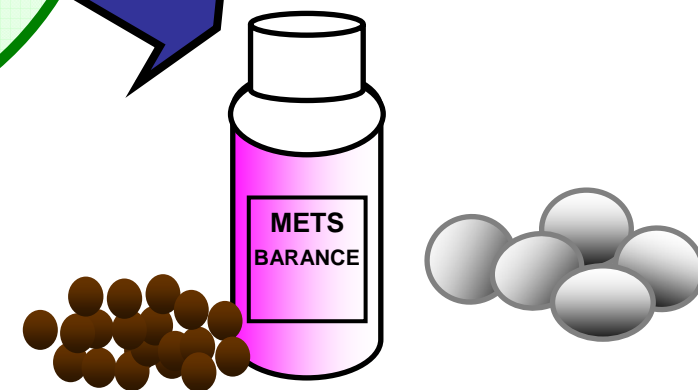
海藻の応用



富栄養水質の 浄化生物



健康食品、化粧品



医学、薬学への応用 (アルギン酸等の硫酸化多糖類)

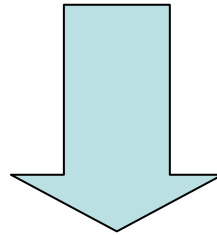
海藻由来の酵素の研究は進んでいない！

理由: 1. 採集が困難

2. 人工増殖が困難

3. 藻体中の大量の多糖類、ポリフェノール類が生化学的解析を阻害

海藻が生産する物質の潜在的な機能は未だ未解明な点が多い！



海藻由来の酵素や機能性物質は新規でしかも実用性が高い可能性を秘めている！

海藻、海草とは？

海藻

(marine algae)

藻類

緑藻類



褐藻類



紅藻類



海草

(seagrass)

被子植物

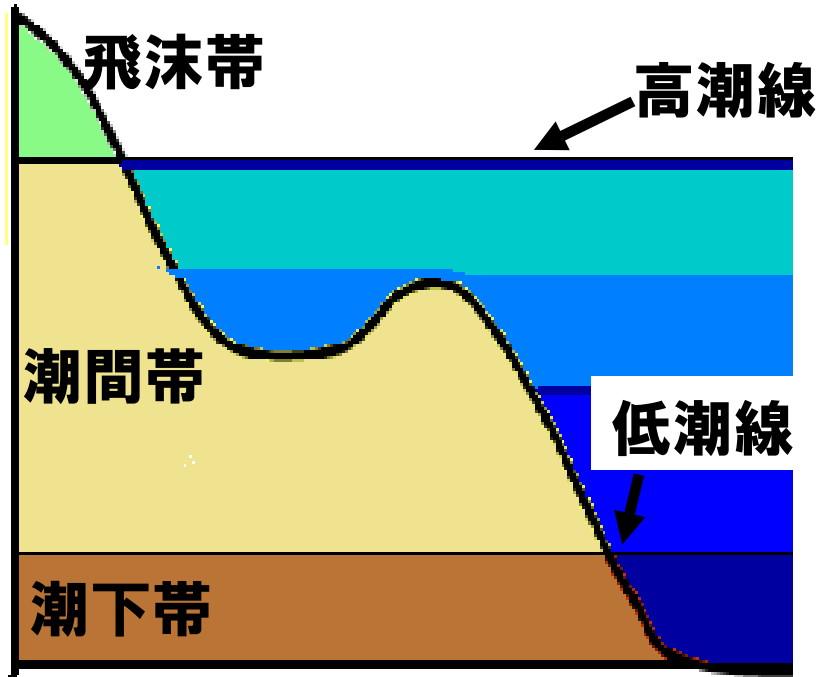


生理活性物質の研究が盛ん

生態分布に関する研究報告が主

しかしながら海藻、海草由来酵素についての研究報告は少ない！！

海藻類の生育帯について



- 潮間帯では通常1日2回の潮の干満により環境が激変。
- 潮間帯に住む海藻は、乾燥や紫外線に対する耐性が高い。

海藻類は様々なストレスにさらされていることから
海藻類由来の酵素はユニークな特徴を示すのではないか!?

当研究室で研究対象にした海藻(草)由来酵素

酵素名

由来

ハロペルオキシダーゼ

Corallina pilulifera(紅藻、ピリヒバ)

Ascophyllum nodosum(褐藻、和名なし)

Ulvella lens(緑藻、アワビモ)

Codium fragile(緑藻、ミル)

キチナーゼ

Phyllospadix japonica(海草、エビアマモ)

ホスファターゼ

Phyllospadix japonica(海草、エビアマモ)



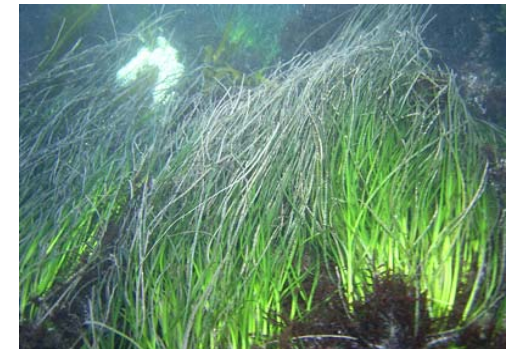
C. pilulifera



A. nodosum



C. fragile



P. japonica

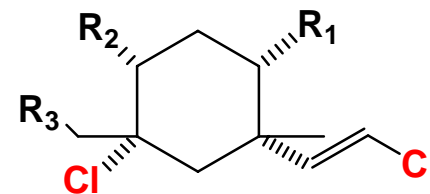
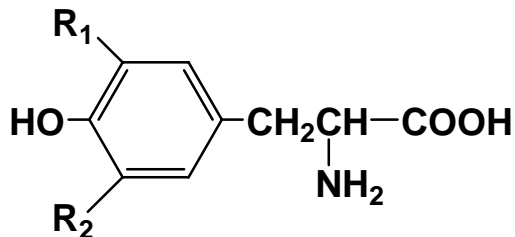
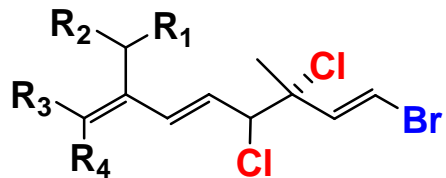
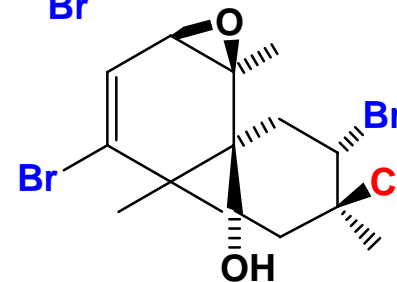
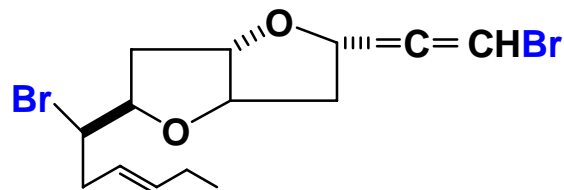
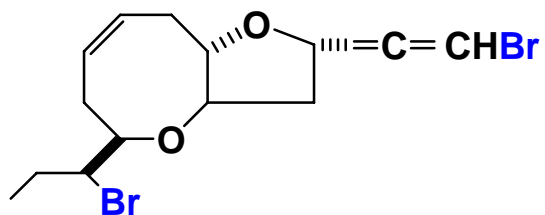
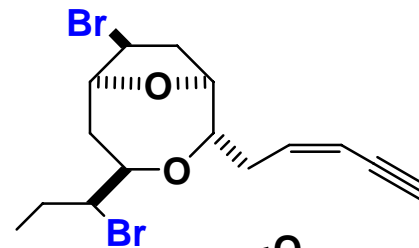
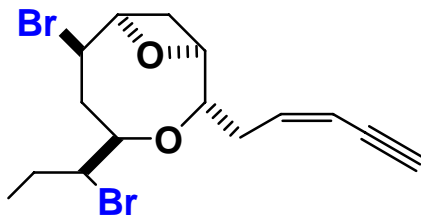
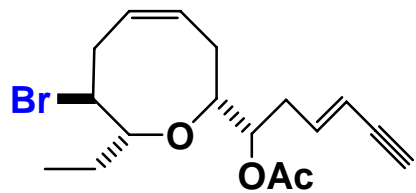
1. ピリヒバ由来ハロペルオキシダーゼ

(I) 酵素の精製、構造解析

(II) 安定性とカルシウム

(III) 変異酵素のハライドに対する活性

2. 緑藻(アワビモ、ミル)由来ハロペルオキシダーゼ



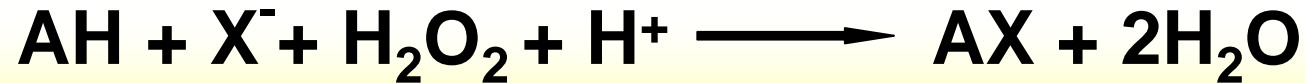
R ₁	R ₂	R ₃	R ₄
H	H	H	Br
H	H	Br	Br
Cl	Cl	H	Cl
Cl	Cl	Cl	H

R ₁	R ₂
H	

R ₁	R ₂	R ₃
Br	Br	H
Cl	Cl	Br
Cl	Br	Br

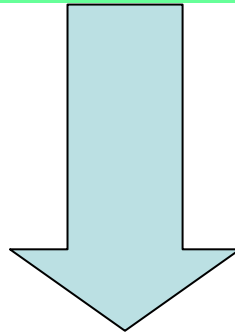
Halogenated Compounds Produced by Algae

Haloperoxidase



(A: nucleophilic cpds. ; X: Cl, Br, I)

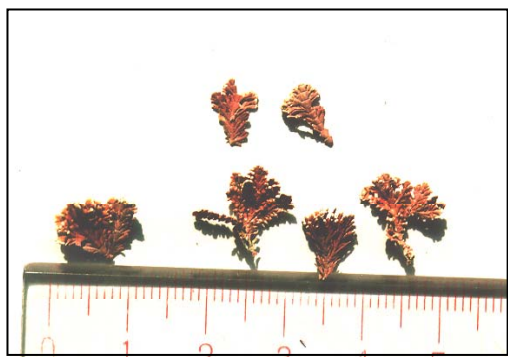
多くの海藻がハロペルオキシダーゼを有し、高活性



海藻由来の酵素の中では
最も研究が進んでいる酵素

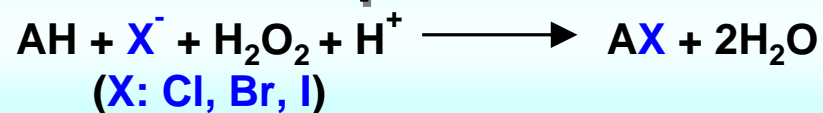
Haloperoxidases Produced by Marine Algae and Microorganisms

Source	Prosthetic group	Halide	Source	Prosthetic group	Halide
Algae			Microorganisms		
brown algae			Fungi		
<i>Ascophyllum nodosum</i>	vanadium	Br, I	<i>Caldariomyces fumago</i>	ferriprotoporphyrin IX	Cl, Br, I
<i>Fucus distichus</i>	vanadium	Br, I	<i>Curvularia inaequalis</i>	vanadium	Cl, Br, I
<i>Laminaria saccharina</i>	vanadium	Br, I	<i>Embellisia didymospora</i>	vanadium	Cl, Br, I
<i>Macrocystis pyrifera</i>	vanadium	Br, I	Bacteria		
red algae			<i>Pseudomonas pyrocinia</i>	ferriprotoporphyrin IX	Br, I
<i>Corallina pilulifera</i>	Vanadium	Br, I	<i>Pseudomonas pyrocinia</i>	zinc and iron	Cl, Br, I
<i>Laurencia japonica</i>	(ferriprotoporphyrin)	Br, I	Actinomycetes		
green algae			<i>Streptomyces aureofaciens</i>	unknown	Br, I
<i>Penicillus capitatus</i>	ferriprotoporphyrin IX	Cl, Br, I	<i>Streptomyces phaeochromogenes</i>	unknown	Br, I
<i>Ulvella lens</i>	(vanadium)	Br, I			

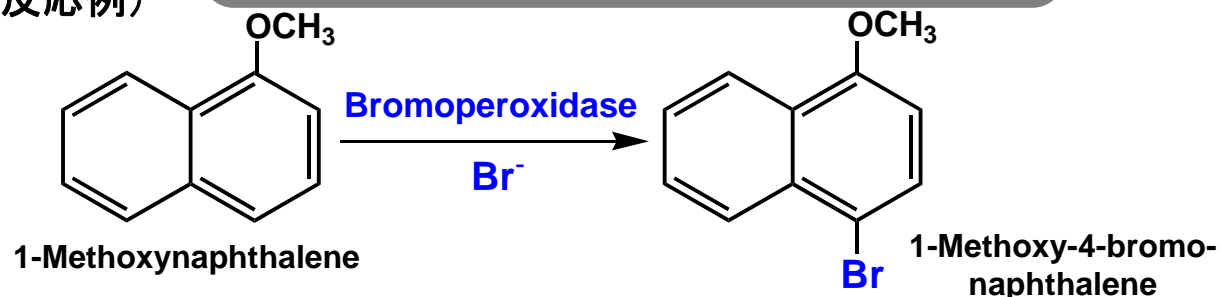


Corallina pilulifera

Haloperoxidase



(反応例)





Corallina pilulifera (ピリヒバ)



鳥取空港付近の海岸

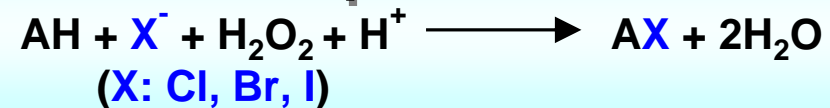
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<i>Laminaria saccharina</i>	vanadium	Br, I	<i>Embellisia didymospora</i>	vanadium	Cl, Br, I
<i>Macrocystis pyrifera</i>	vanadium	Br, I	Bacteria		
red algae			<i>Pseudomonas pyrocinia</i>	ferriprotoporphyrin IX	Br, I
<i>Corallina pilulifera</i>	Vanadium	Br, I	<i>Pseudomonas pyrocinia</i>	zinc and iron	Cl, Br, I
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<i>Penicillus capitatus</i>	ferriprotoporphyrin IX	Cl, Br, I	<i>Streptomyces phaeochromogenes</i>	unknown	Br, I
<i>Ulvella lens</i>	(vanadium)	Br, I			



Ascophyllum nodosum

Haloperoxidase





大堤防 Afsluitdijk(オランダ北部)



Ascophyllum nodosum の採集

1. ピリヒバ由来ハロペルオキシダーゼ

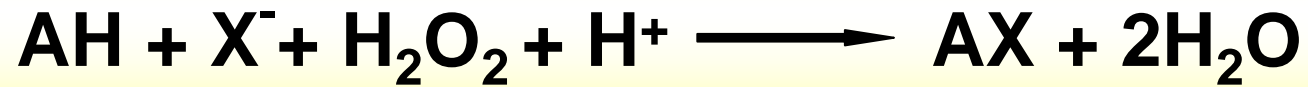
(I) 酵素の精製、構造解析

(II) 安定性とカルシウム

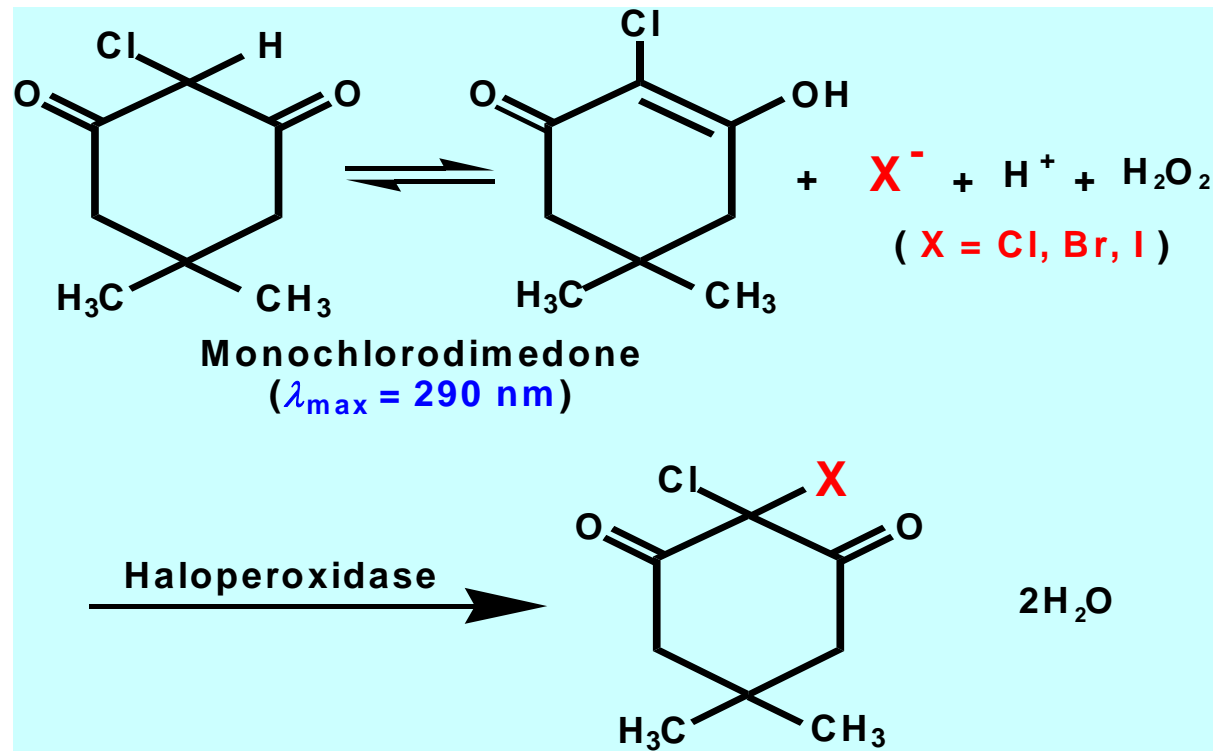
(III) 変異酵素のハライドに対する活性

2. 緑藻(アワビモ、ミル)由来ハロペルオキシダーゼ

Haloperoxidase



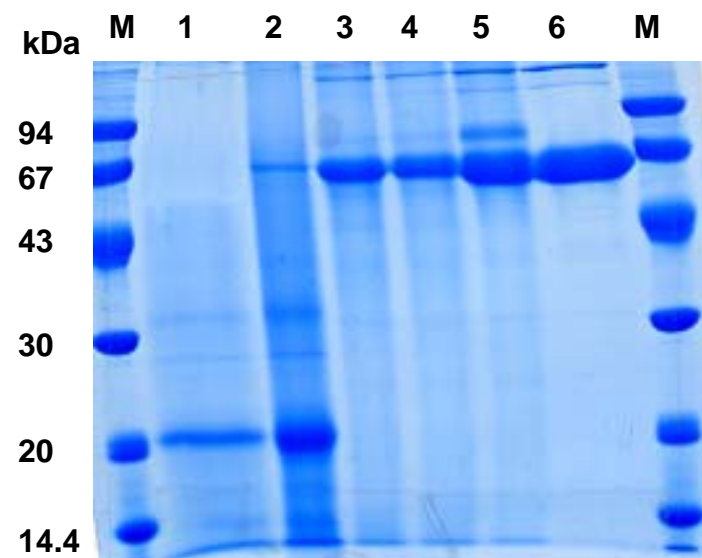
(A: nucleophilic cpds. ; X: Cl, Br, I)



Purification of BPO Produced by *Cor. pilulifera*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	35,000	4,240	8.25	1	100
(NH ₄) ₂ SO ₄ fractionation	35,700	4,230	8.44	1.02	102
DEAE-Sepharose	22,600	188	120	14.5	64.6
1st Q-Sepharose	9,230	57.6	160	19.4	26.4
Sepharose CL-4B	7,360	25.9	284	34.4	21.0
2nd Q-Sepharose	4,560	11.6	393	47.6	13.0

SDS-PAGE of BPO from *Cor. pilulifera*



- M. Marker proteins
1. Cell-free extract
 2. (NH₄)₂SO₄ fractionation
 3. DEAE-Sepharose
 4. 1st Q-Sepharose
 5. Sepharose CL-4B
 6. 2nd Q-Sepharose

Comparison of Properties between BPOs from *Cor. pilulifera* and *A. nodosum*

	<i>Cor. pilulifera</i>	<i>A. nodosum</i>
Molecular weight	680 kDa	90 kDa
Subunit weight	64 kDa × 12	40 kDa x 2
Optimum temp.	65°C	N.D*
Optimum pH	6	6.5
Heat stability	85°C (90% 20 min)	70°C (38%, 20 min)
pH stability	5-13	N.D*
Specific activity	393 U/mg (BPO)	87.5 U/mg (BPO)
<i>K_m</i> value	0.12 mM (H ₂ O ₂) 8.4 mM (KBr)	12.7 mM (KBr)

*Not determined

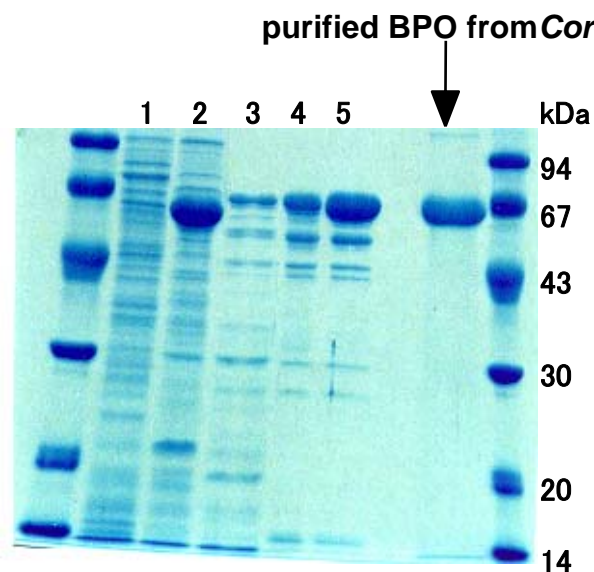
BPO1	MGIPADNLQSRKASFDTRVAAAELALNRGVVPSFANGEELLYRNPDNDTPSFASF	60
BPO2A.....CETG--.....	58
BPO1	KGLPHDDNGAIIDPDDFLAFVRAINSGDEKEIADLTGPARDPETGLPIWRSDLANSLEL	120
BPO2	118
BPO1	EVRGWENSSAGLTFDLEGPDAQSIAMPPAPVLTSPELVAEIAELYLMALGREIEFSEFDS	180
BPO2V.....M...I.....D.....	178
BPO1	PKNAEYIQFAIDQLNGLEWFNTPAKLGDPPAEIRRRRGEVTVGNLFRGILPGSEVGPYLS	240
BPO2AF.RS..ER.....	238
BPO1	QYIIVGSKQIGSATVGNKTLVSPNAADEFDGEIAYGSITISQVRRIATPGRDFMTDLKVF	300
BPO2F.....	298
BPO1	LDVQDAADFRGFESYEPGARLIRTIRDLATWVHFDALYEAYLNAACLILLANGVPFDPNLP	360
BPO2G.....	358
BPO1	FQQEDKLDNQDVFVNFVFGSAHVLSLVTEVATRALKAVRYQKFNIHRRRLRPEATGGLISVNK	420
BPO2H.....	418
BPO1	IAPQKG-ESIFPEVDLAVEELGDILEK-AEISNRKQNIADGDPDPDPSFLLPMAFAEGSP	478
BPO2	.KSFLA.SDI.....SEL.....SS..DDV...-..E...R...IVS..K.....	477
BPO1	FHPSYSGSHAVVAGACVTILKAFFDSGIEIDQVFEVDKDEDKLVKSSFKGTLTVAGELNK	538
BPO2ANFQ..K.....T.....	537
BPO1	LADNIAIGRNMAGVHYFSDQFESLLLGEQVAIGILEEQSLTYGENFFFNLPKFDGTTIQI	598
BPO2V.....I.....	597

**Alignment of the Amino Acid Sequences of BPOs,
BPO1 and BPO2 from *Cor. pilulifera***

	Protein (mg)	Total activity (U)	Specific activity (U/mg)
1. Cell-free extracts	17600	—	—
2. DEAE-Sepharose	264	613	2.32
3. Phenyl-Sepharose	9.92	363	36.6
4. Source 30Q	1.66	159	95.9
5. Superdex	0.341	97.5	286

発現量低い

無細胞抽出液中で
活性測定できない



N-Terminal amino acid sequence

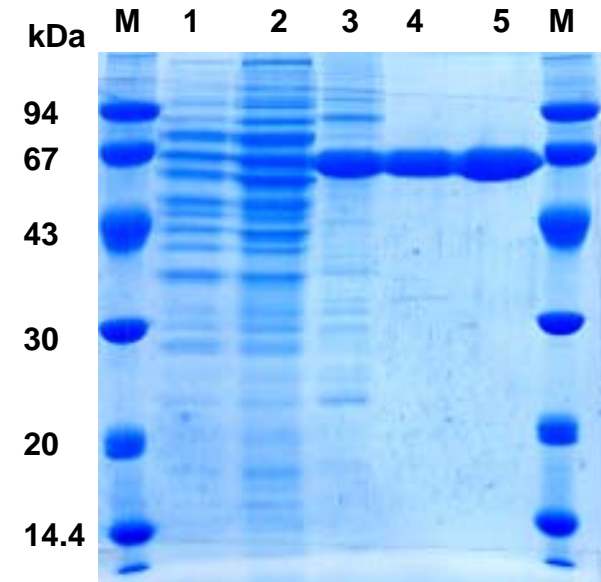
G-I-P-A-D-N-L-Q-S-R-A-K-A-S-F-D

Purification of BPO from the Recombinant *E. coli*

Purification of BPO Produced by *Saccharomyces cerevisiae* BJ1991/pTNT30

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	225,000	10,300	21.8	1	100
(NH ₄) ₂ SO ₄ fractionation	213,000	5,730	37.2	1.71	94.7
DEAE-Sepharose	160,000	627	255	11.7	71.1
Q-Sepharose	65,700	162	406	18.6	29.2
Sepharose CL-4B	63,000	146	432	19.8	28.0

SDS-PAGE of BPO from *S. cerevisiae* BJ1991/pTNT30



M. Marker proteins

1. Cell-free extract

2. (NH₄)₂SO₄ fractionation

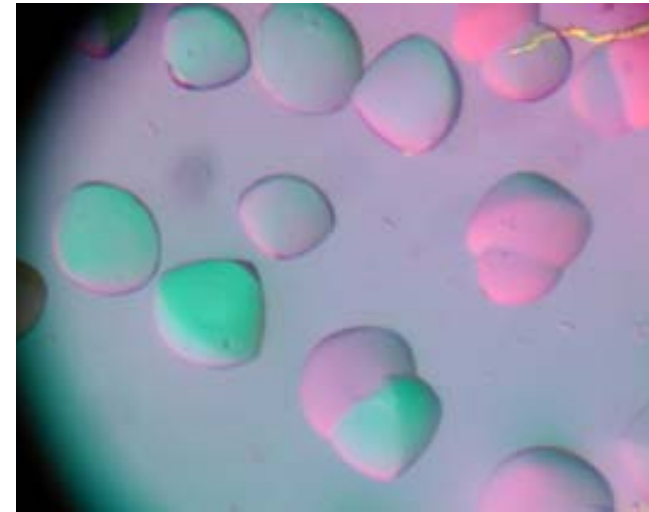
3. DEAE-Sepharose

4. Q-Sepharose

5. Sepharose CL-4B

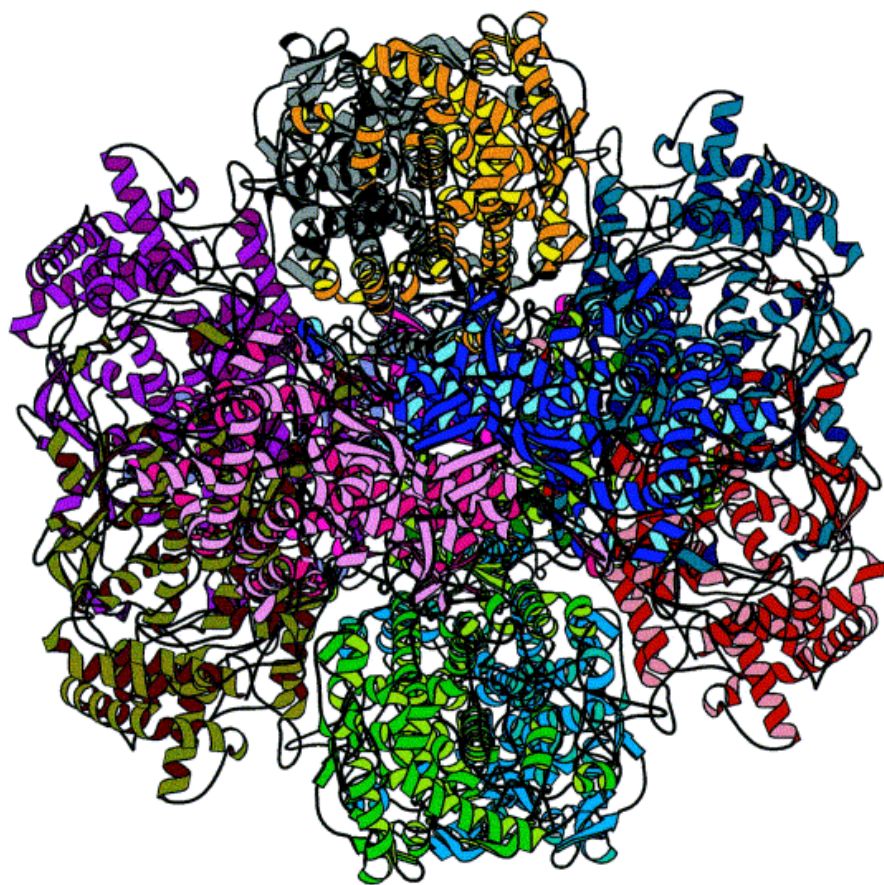


1.8 M Ammonium dihydrogenphosphate
0.1 M Tris-HCl buffer, pH 5.0 (1 day)

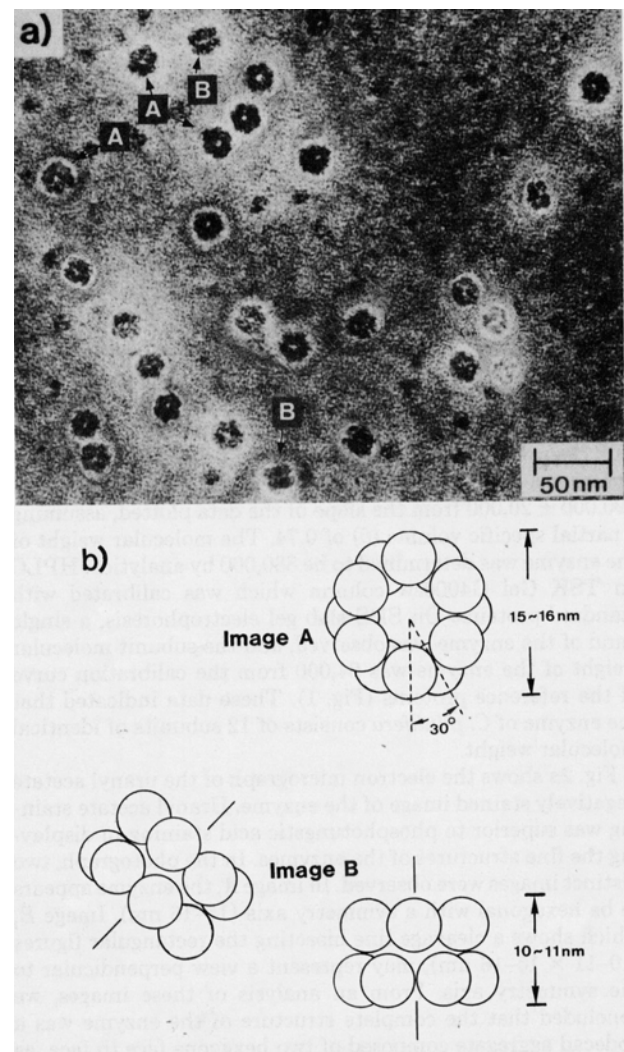


1.8 M Ammonium dihydrogenphosphate
0.1 M Tris-HCl buffer, pH 5.0 (6 days)

Photomicrographs of Crystalline BPO from *S. cerevisiae* BJ1991/pTNT30



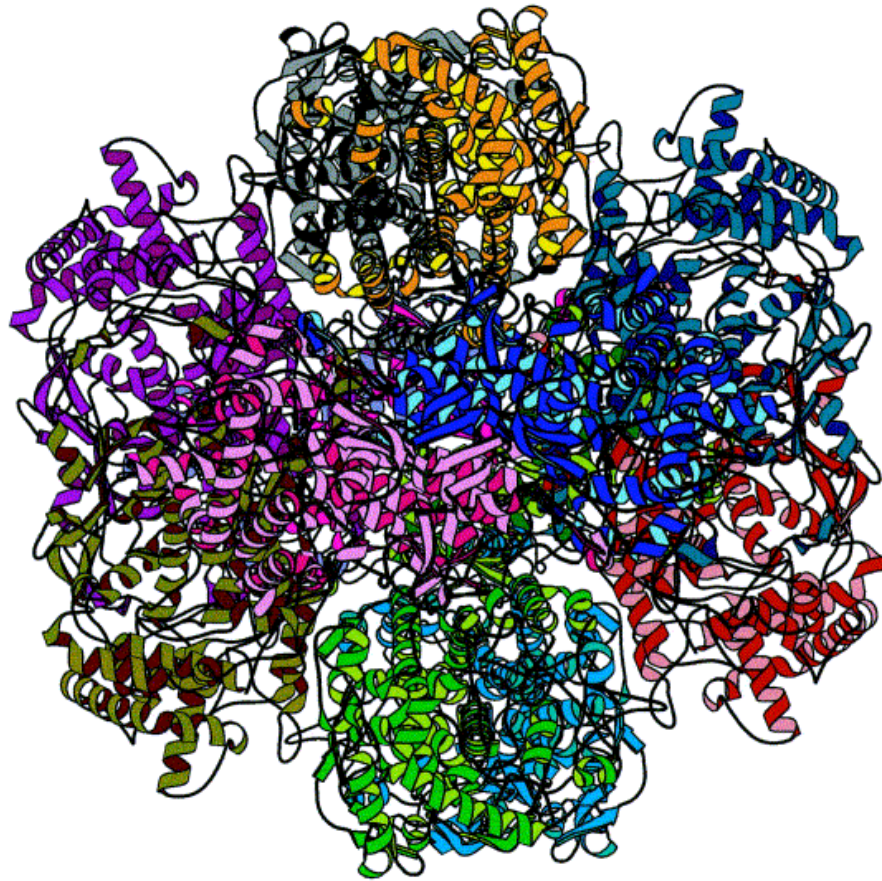
Coordination Chemistry Reviews, 237, 65-76 (2003)



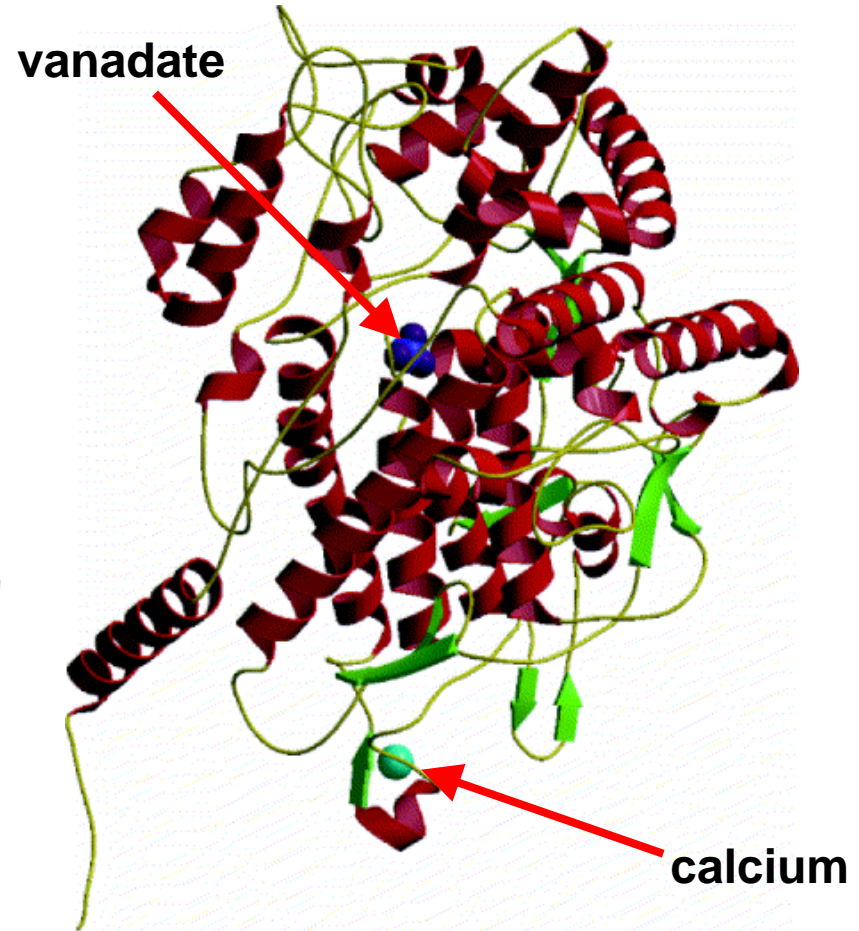
Electron Micrograph

J.B.C., 261, 5194-5200 (1986)

Dodecamer Structure of the *Cor. pilulifera* BPO



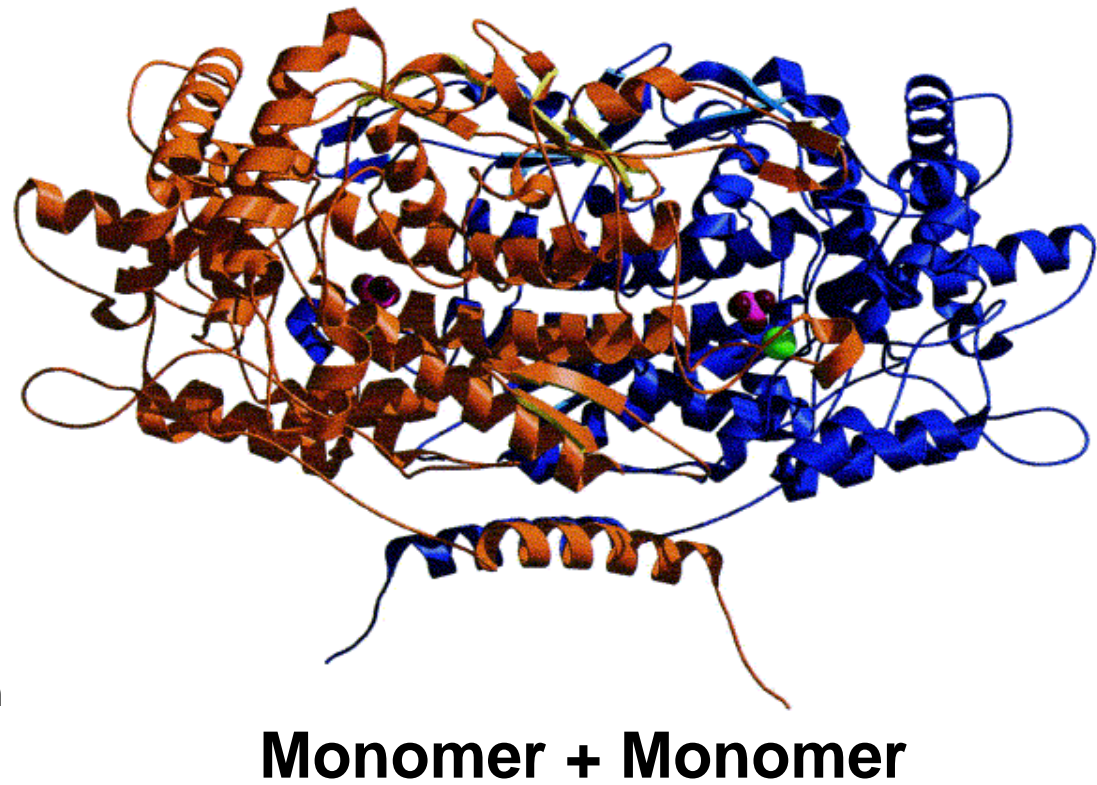
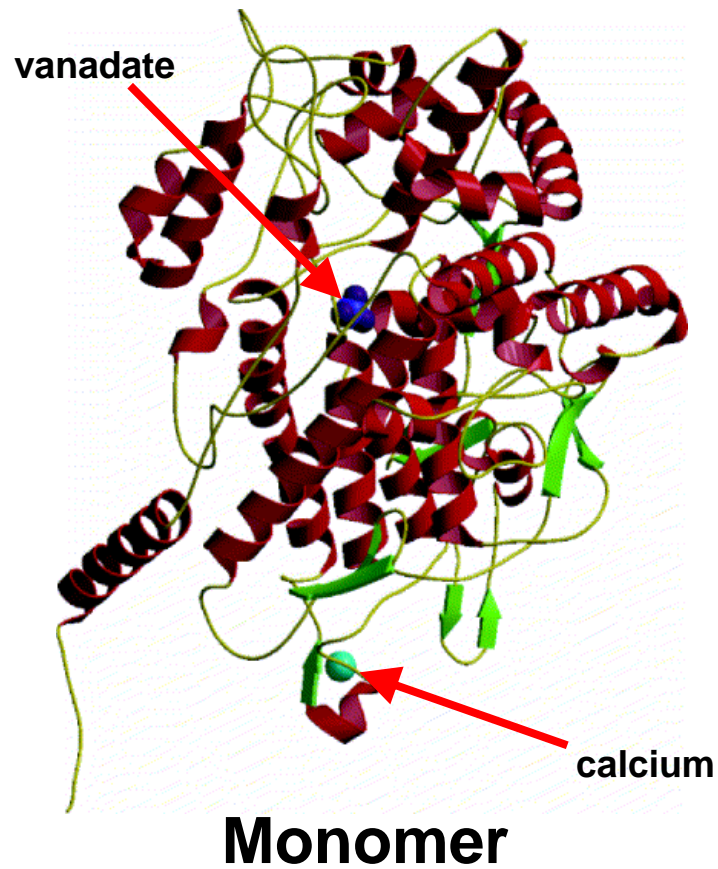
Dodecamer



Monomer

Structure of the *Cor. pilulifera* BPO

バナジン酸が位置する活性中心から離れた場所にカルシウムが存在



Structure of the *Cor. pilulifera* BPO

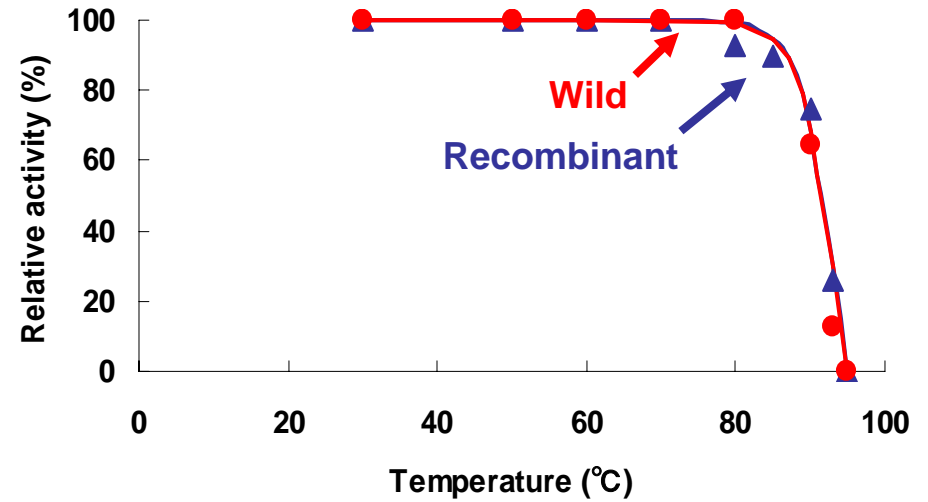
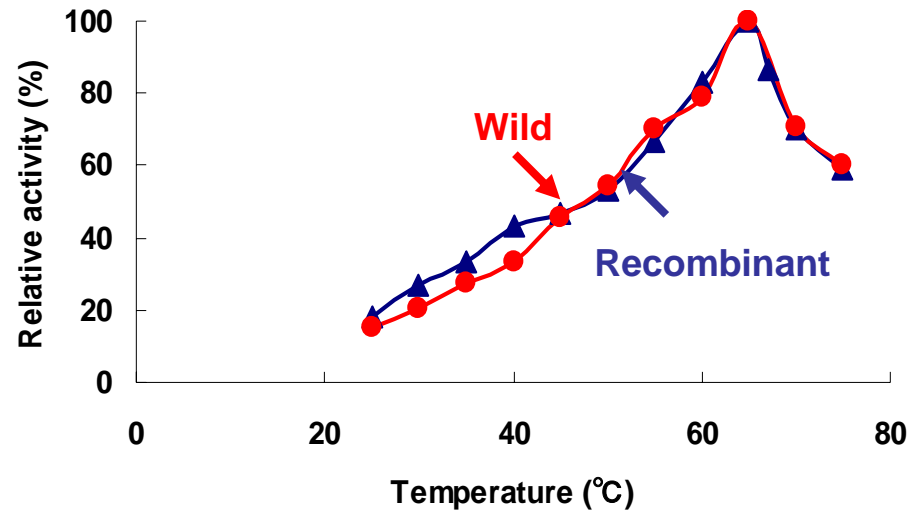
1. ピリヒバ由来ハロペルオキシダーゼ

(I) 酵素の精製、構造解析

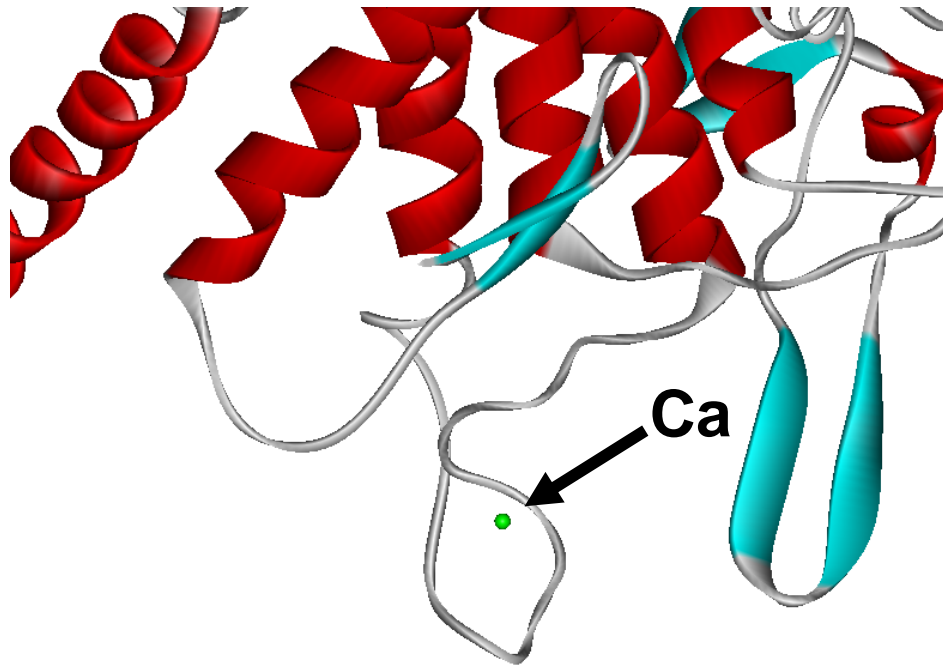
(II) 安定性とカルシウム

(III) 変異酵素のハライドに対する活性

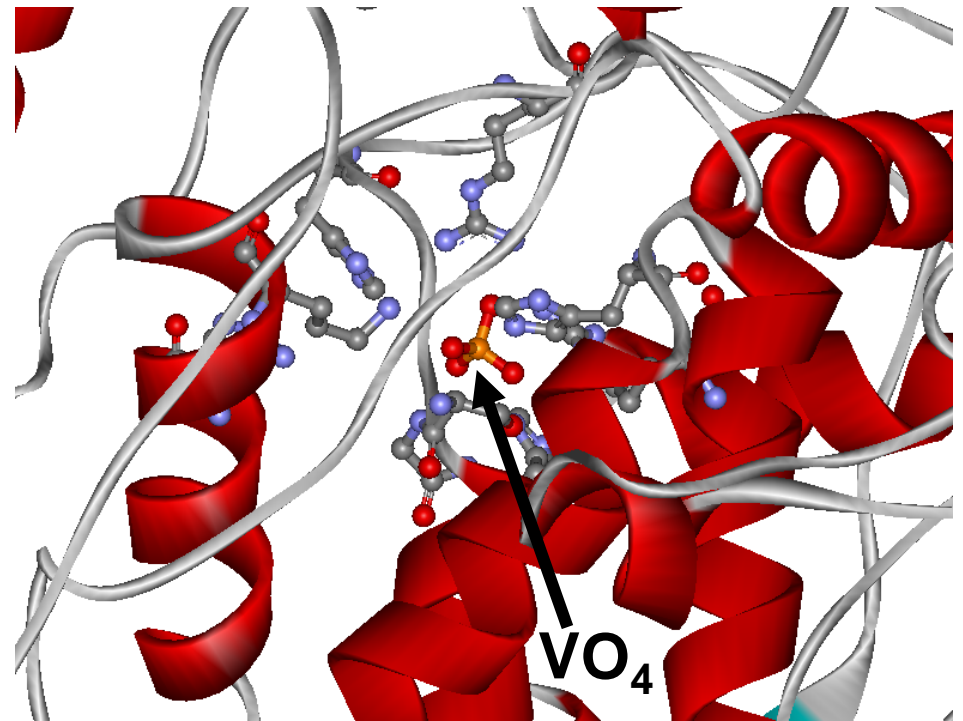
2. 緑藻(アワビモ、ミル)由来ハロペルオキシダーゼ



Effects of Temperature on Wild or Recombinant BPO Activity and Stability



Structure of the Calcium Binding Site of BPO from *Cor. pilulifera*



Structure of the Vanadate Binding Site of BPO from *Cor. pilulifera*

Determination of Calcium in BPO by Inductively Coupled Plasma (ICP) Emission Spectrometry

	Wild	Recombinant	Apo	
			Wild	Recombinant
mol Ca/mol subunit	0.976	0.828	N.D.	0

N.D. : not determined

Enzymes from *Cor. pilulifera* and the recombinant yeast

- dialyzed against 10 mM EDTA/
100 mM citric acid-potassium dihydrogenphosphate buffer
(pH 3.8) for 24 h
- dialyzed against 50 mM Tris-SO₄ buffer (pH 7.4) for 24 h

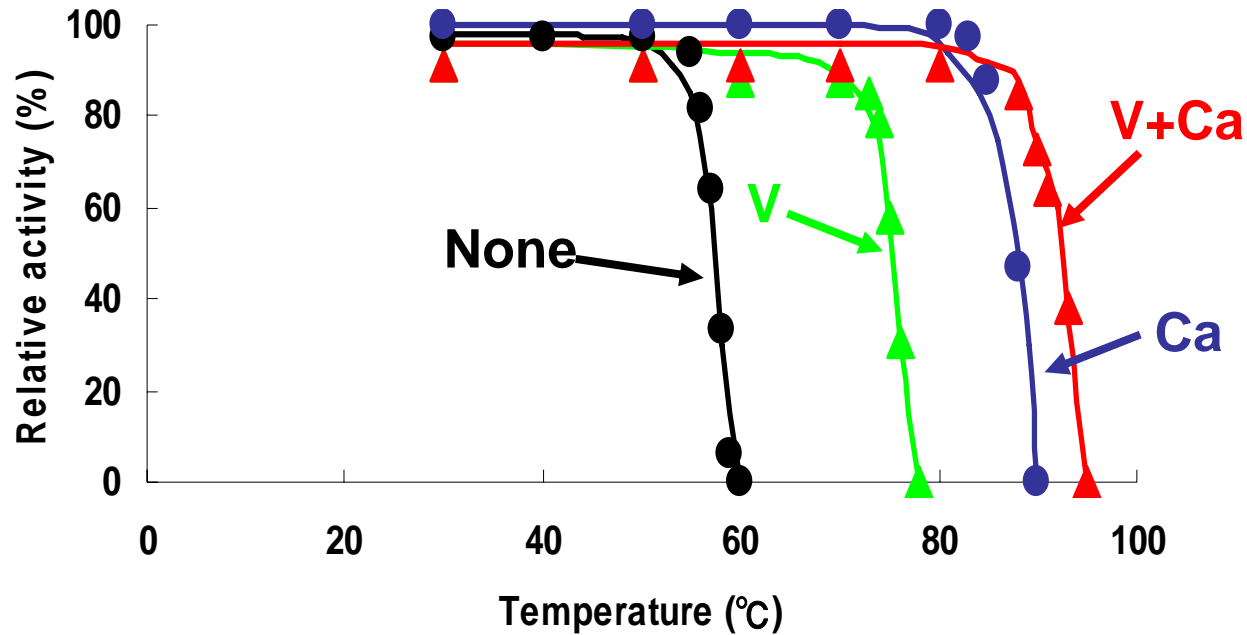
Apo-enzymes

- preincubated with 1 mM Na₃VO₄ and/or 1 mM various metal salts
at 30°C for 12 h

Holo-enzymes

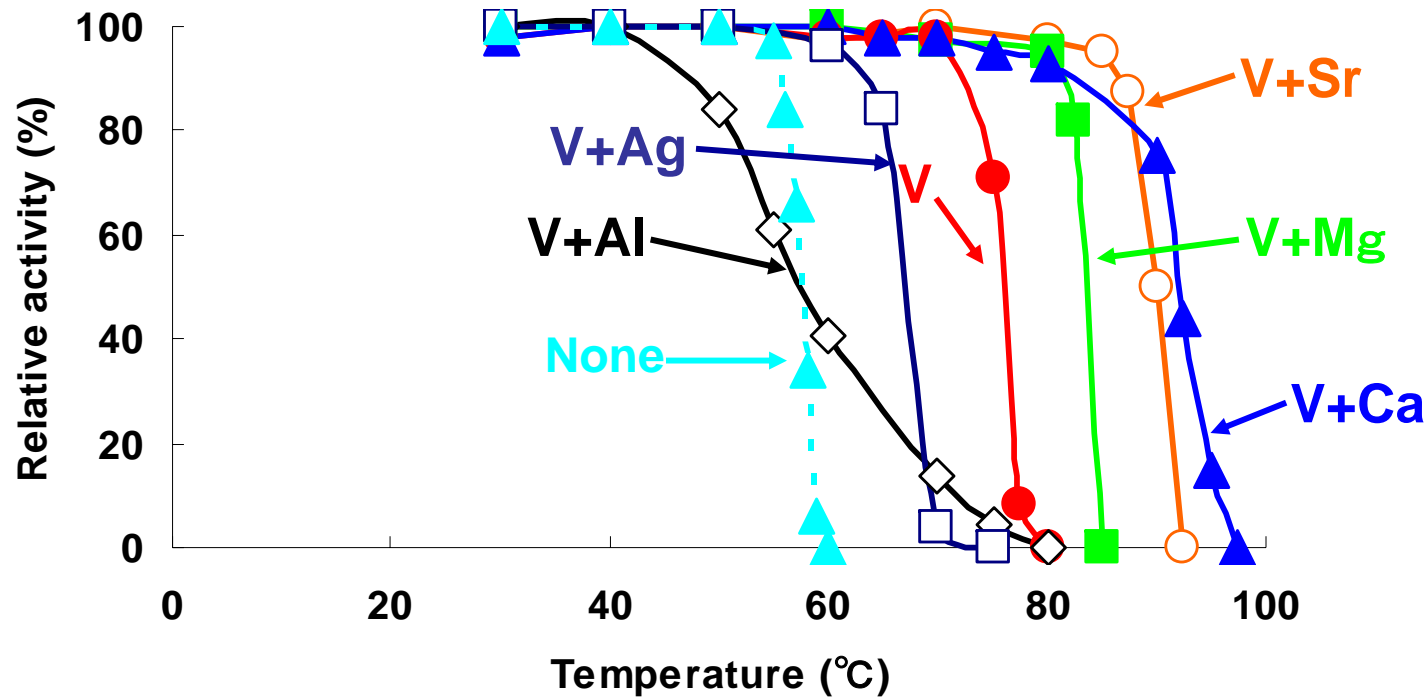
- heat treatment (20 min)
- measure BPO activity

Scheme of Formation of Apo-enzyme and Holo-enzyme



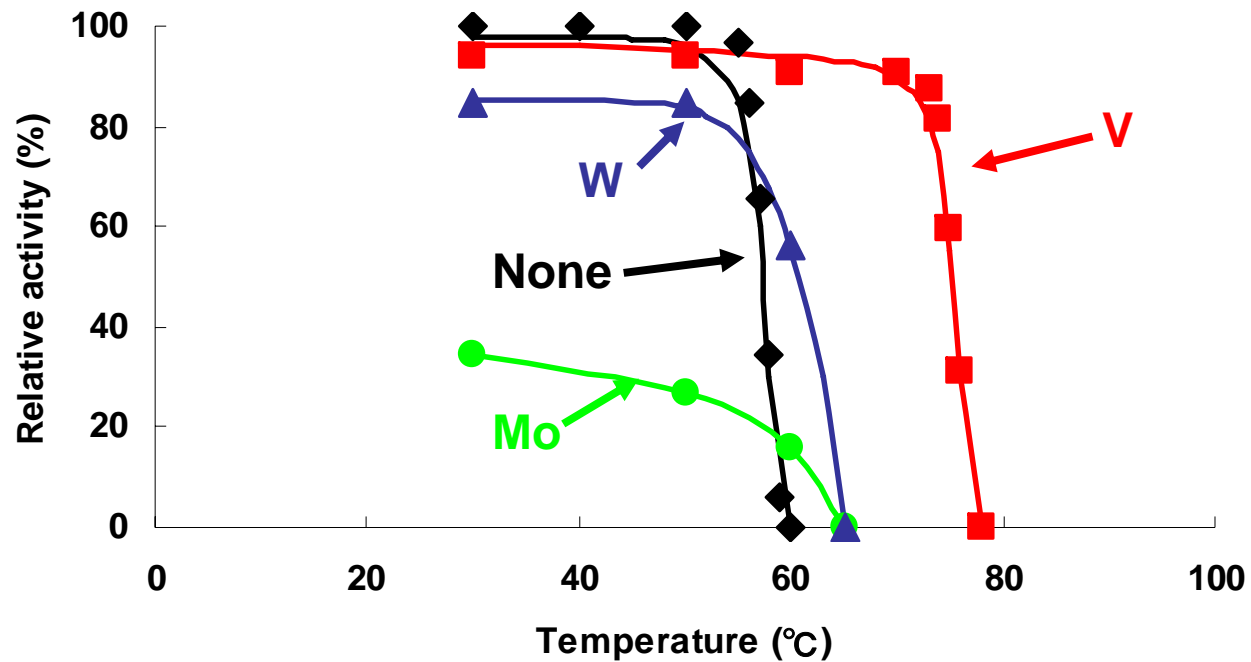
Effects of Vanadate and Calcium Ions on Thermostability of Apo-BPO

Ca が熱安定性に関与、V も多少関与



Effects of Various Metal Ions on Thermostability of Apo-BPO

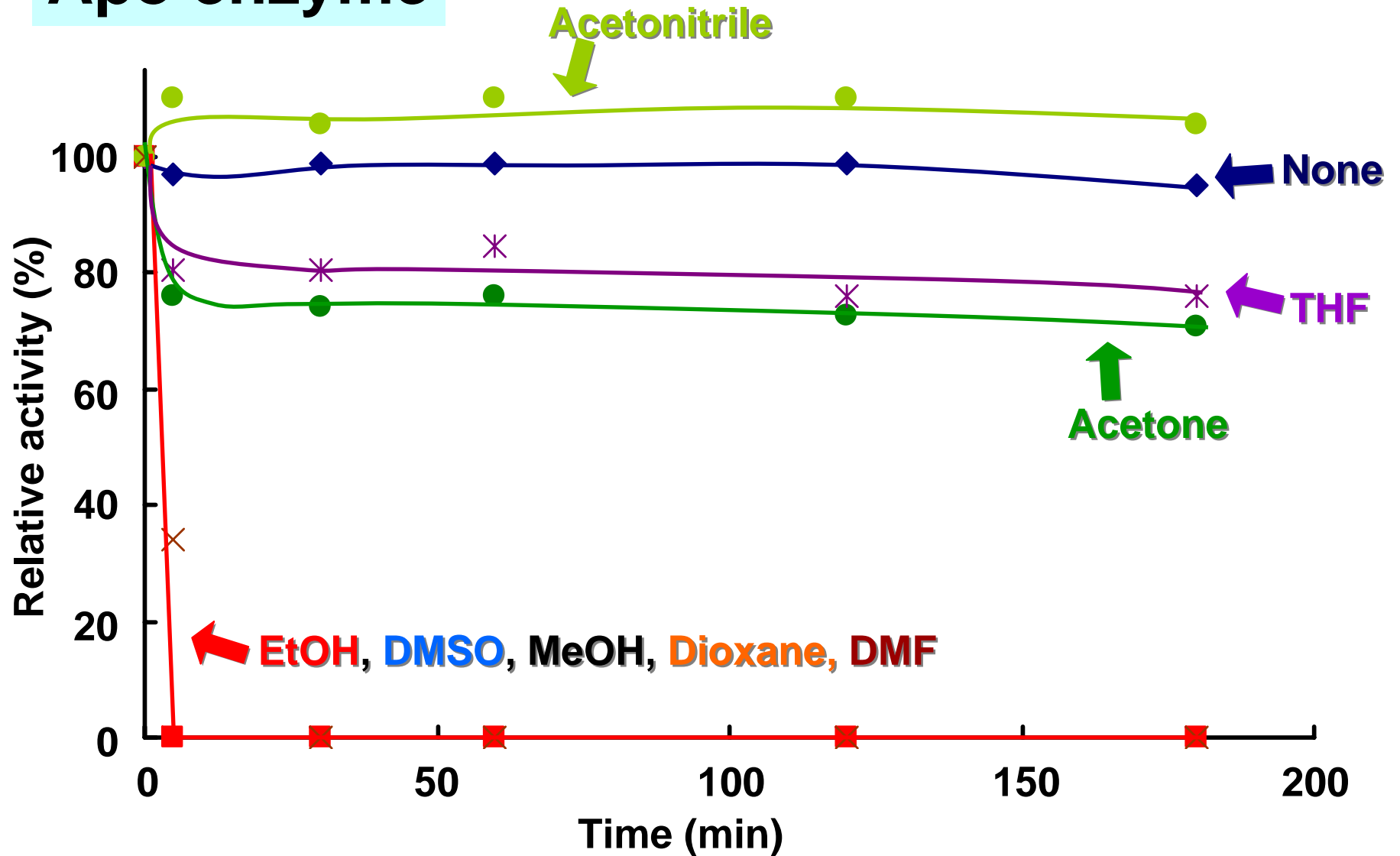
Mg, Sr に Ca の代替効果有り



Effects of Tungstate Ion and Molybdate Ions on Thermostability of Apo-BPO

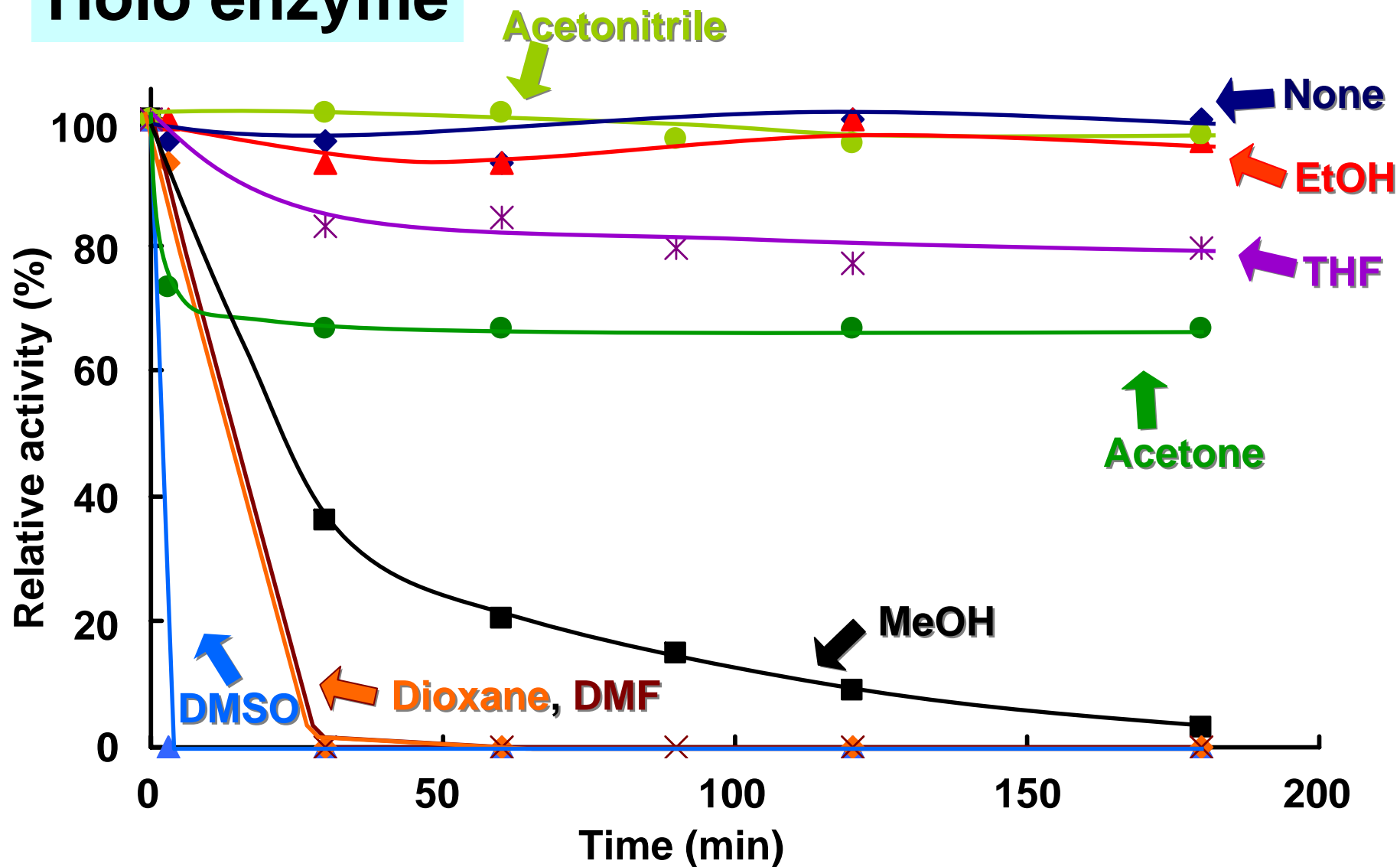


Apo enzyme



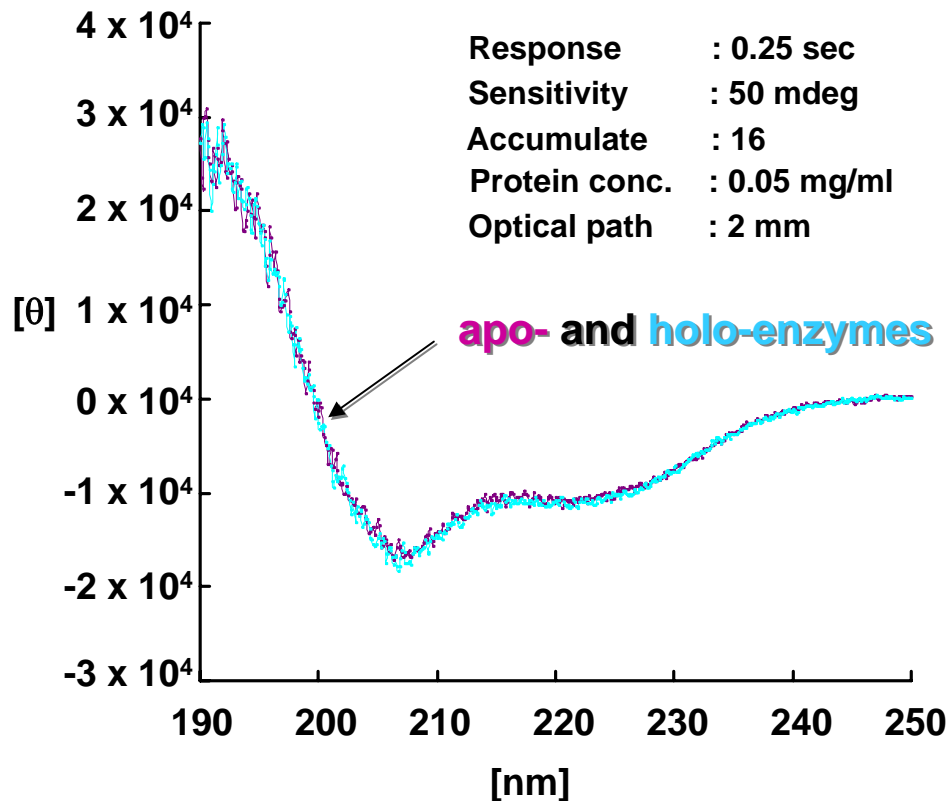
Effects of Polar Organic Solvents on the Stability of Apo-BPO

Holo enzyme

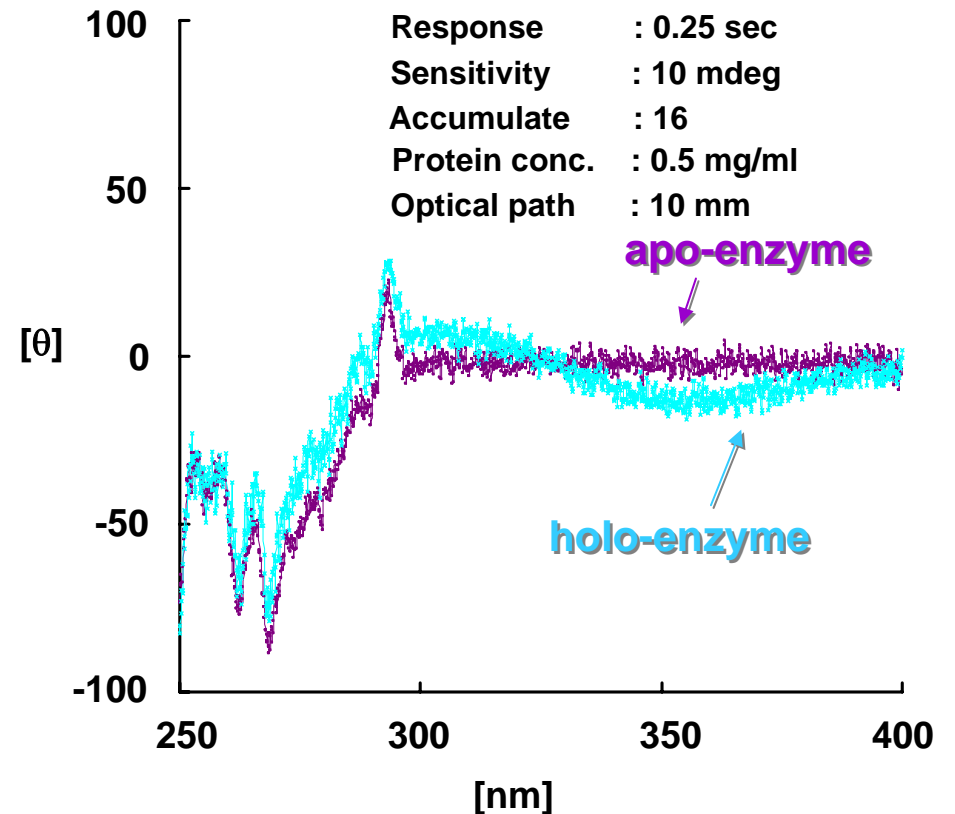


Effects of Polar Organic Solvents on the Stability of Holo-BPO

Far UV



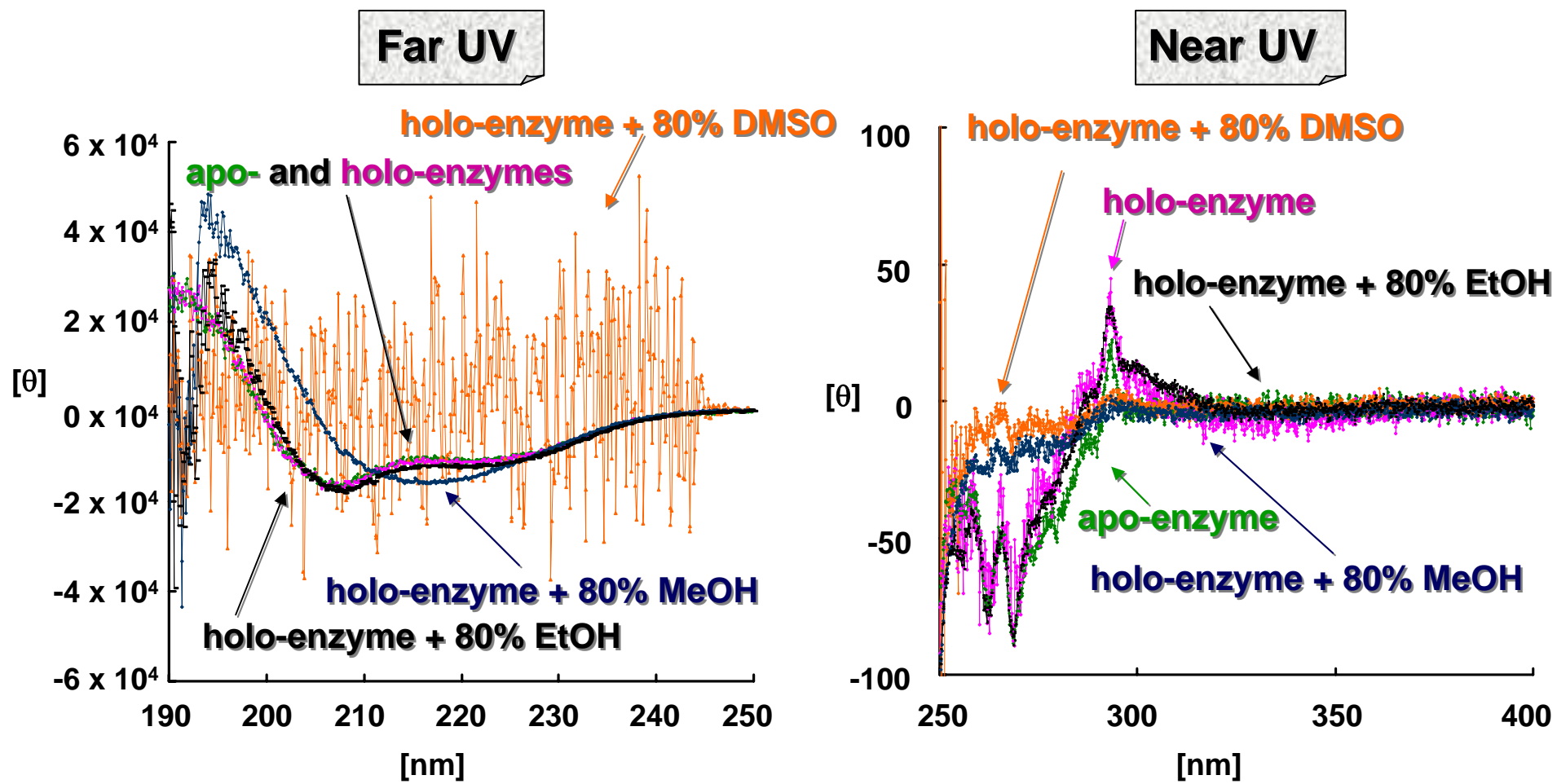
Near UV



Analysis of CD Spectrometry of Apo- and Holo-enzymes

アポ型、ホロ型酵素の CD スペクトルに大差なし

→ アポ型、ホロ型酵素で極端な構造変化なし



Analysis of CD Spectrometry of Holo-enzymes in 80% Organic Solvents

エタノール添加では構造変化なし、
メタノール添加で 195 nm ~ 230 nm の CD 波形にずれ

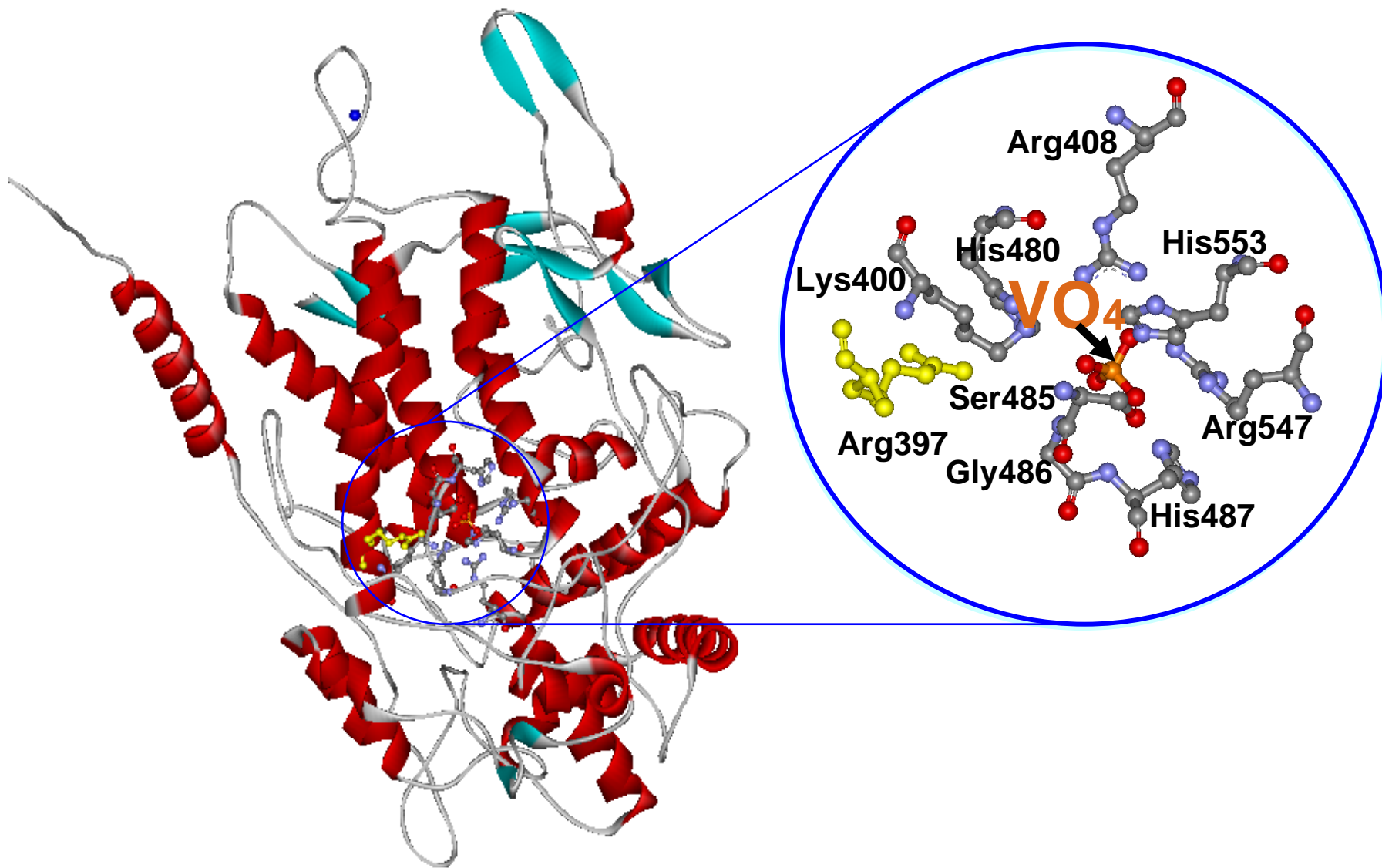
1. ピリヒバ由来ハロペルオキシダーゼ

(I) 酵素の精製、構造解析

(II) 安定性とカルシウム

(III) 変異酵素のハライドに対する活性

2. 緑藻(アワビモ、ミル)由来ハロペルオキシダーゼ

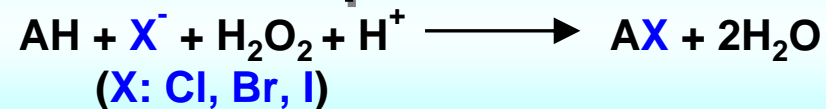


**Structure and Active Site of the BPO Subunit
from *Cor. pilulifera***

Haloperoxidases Produced by Marine Algae and Microorganisms

Source	Prosthetic group	Halide	Source	Prosthetic group	Halide
Algae			Microorganisms		
brown algae			Fungi		
<i>Ascophyllum nodosum</i>	vanadium	Br, I	<i>Caldariomyces fumago</i>	ferriprotoporphyrin IX	Cl, Br, I
<i>Fucus distichus</i>	vanadium	Br, I	<i>Curvularia inaequalis</i>	vanadium	Cl, Br, I
<i>Laminaria saccharina</i>	vanadium	Br, I	<i>Embellisia didymospora</i>	vanadium	Cl, Br, I
<i>Macrocystis pyrifera</i>	vanadium	Br, I	Bacteria		
red algae			<i>Pseudomonas pyrocinia</i>	ferriprotoporphyrin IX	Br, I
<i>Corallina pilulifera</i>	Vanadium	Br, I	<i>Pseudomonas pyrocinia</i>	zinc and iron	Cl, Br, I
<i>Laurencia japonica</i>	(ferriprotoporphyrin)	Br, I	Actinomycetes		
green algae			<i>Streptomyces aureofaciens</i>	unknown	Br, I
<i>Penicillus capitatus</i>	ferriprotoporphyrin IX	Cl, Br, I	<i>Streptomyces phaeochromogenes</i>	unknown	Br, I
<i>Ulvella lens</i>	(vanadium)	Br, I			

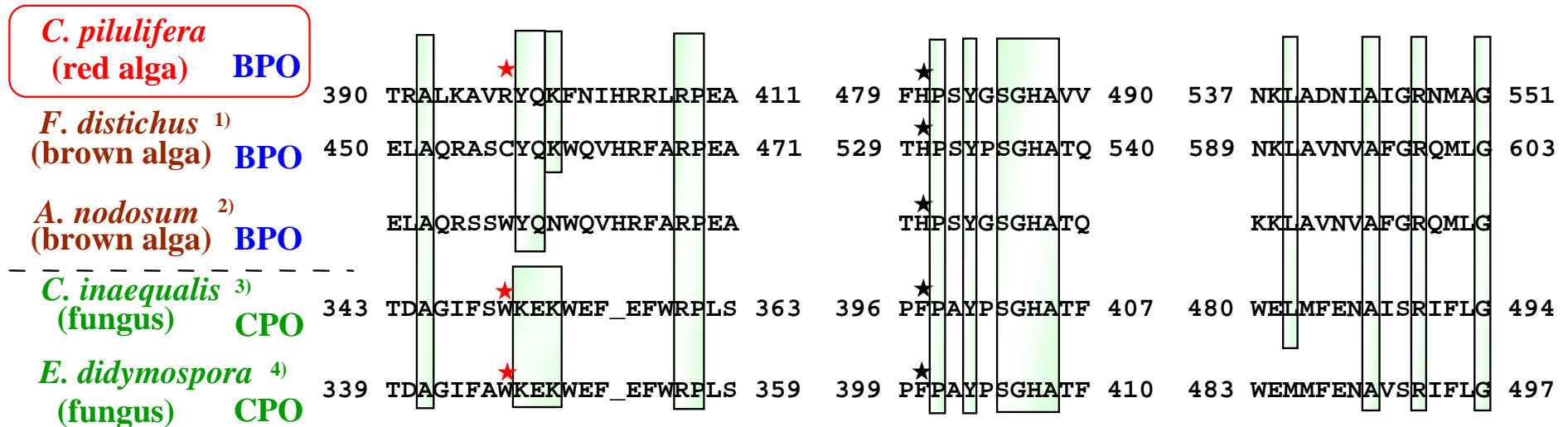
Haloperoxidase



Comparison of Properties between BPO from *Cor. pilulifera* and CPO from *Cur. inaequalis*

	BPO	CPO
Molecular weight	680 kDa	67 kDa
Subunit weight	64 kDa × 12	67 kDa
Optimum temp.	65°C	N.D*
Optimum pH	6	5.5
Heat stability	85°C (90% 20 min)	80°C (73%, 5 min)
pH stability	5-13	N.D*
Specific activity	432 U/mg (BPO) 0.65 U/mg (CPO)	48 U/mg (BPO) 14 U/mg (CPO)
<i>K_m</i> value	0.12 mM (H ₂ O ₂) 8.4 mM (KBr) N.D* (KCl)	1 μM (H ₂ O ₂) 0.25 mM (KCl)

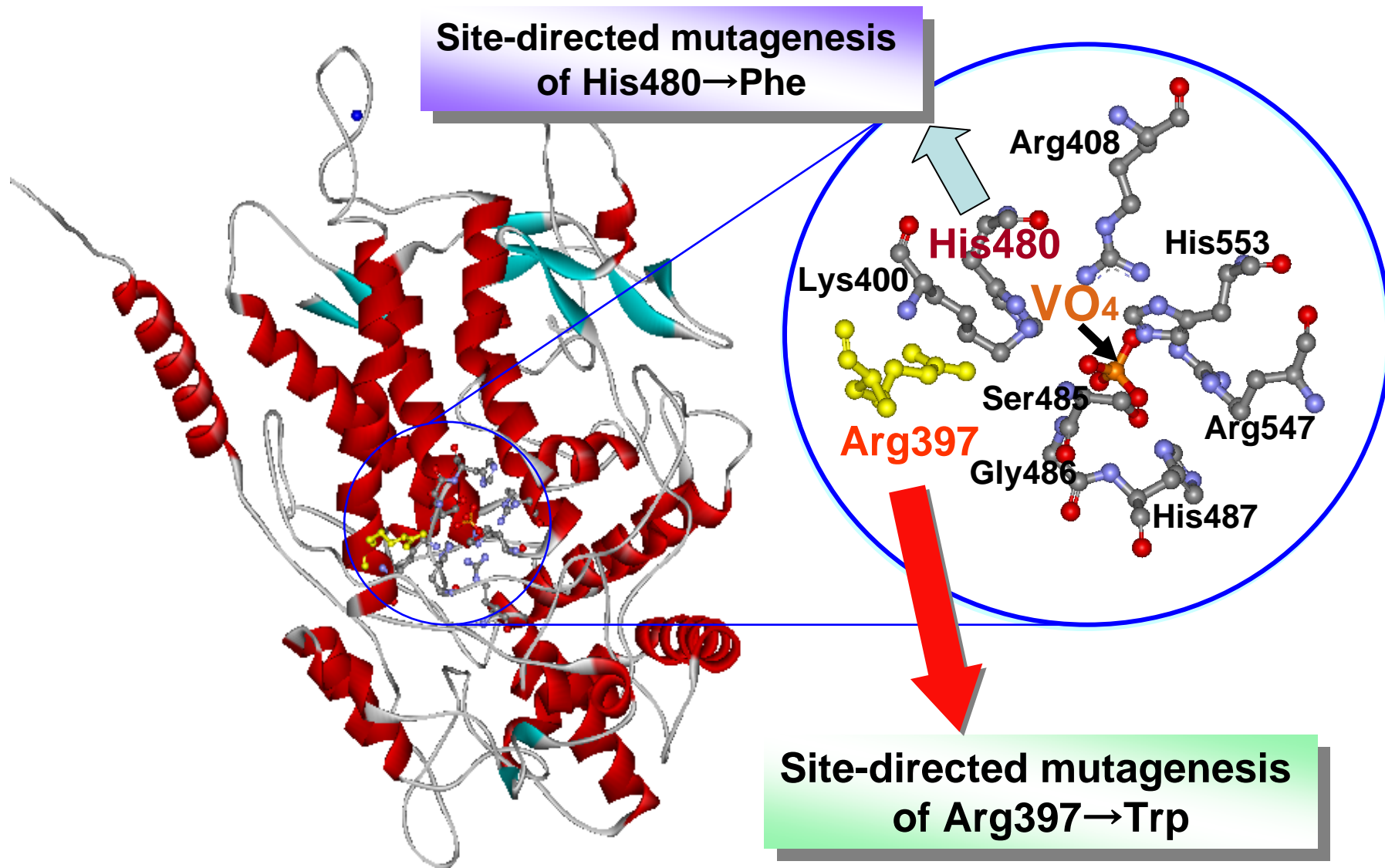
*Not determined



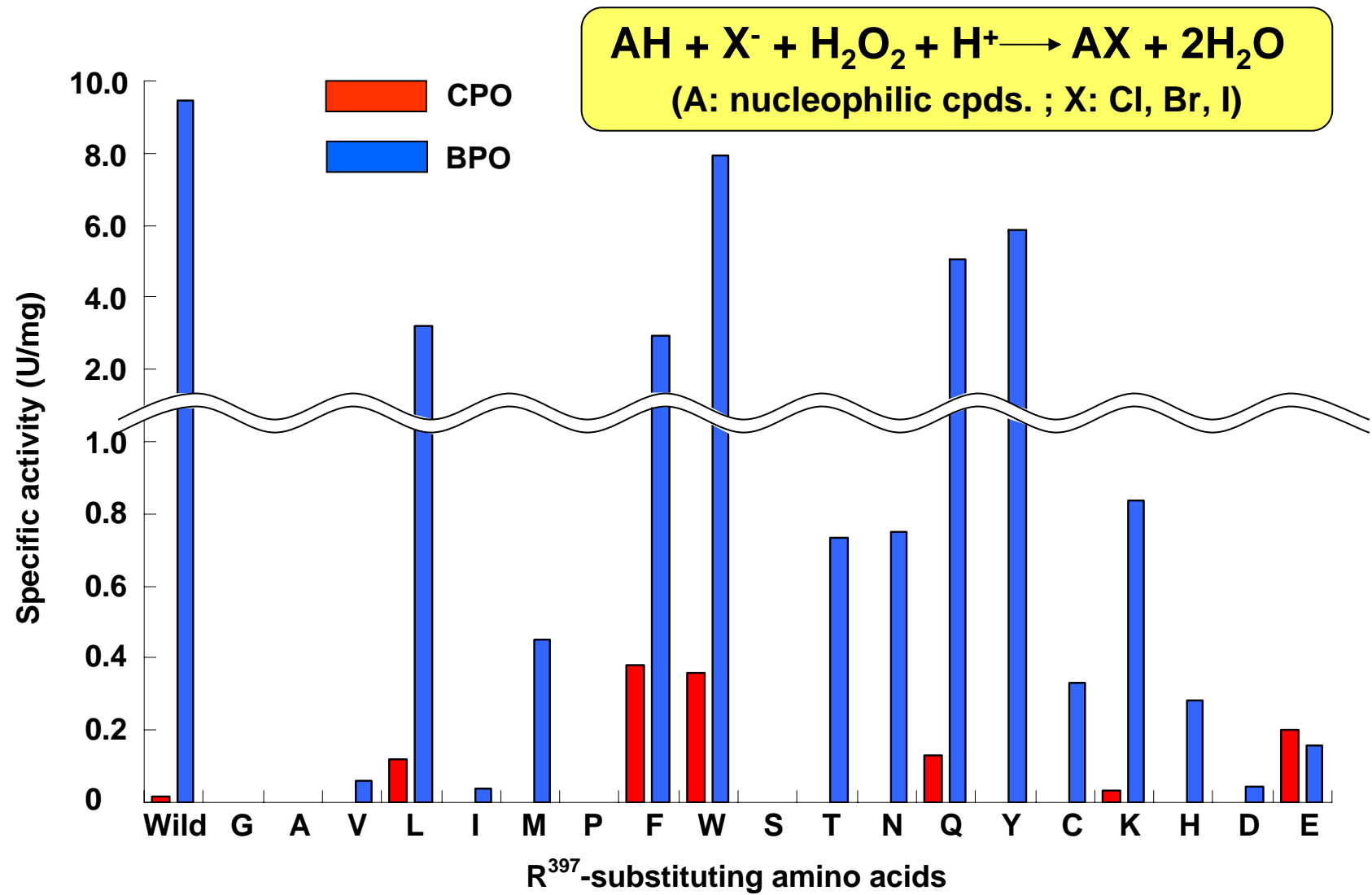
Alignment of the Amino Acid Sequences of Vanadium-dependent Haloperoxidases from Algae and Microorganisms

***C. pilulifera*: Corallina pilulifera, *F. distichus*: Fucus distichus, *A. nodosum*: Ascophyllum nodosum, *C. inaequalis*: Curvularia inaequalis, *E. didymospora*: Embellisia didymospora**

- 1) The sequence was submitted to the GenBank Data Bank under the accession number AF053411 (1998).
- 2) The partial amino acid sequence was determined.
- 3) Eur. J. Biochem., 229 , 566-574 (1995).
- 4) J. Biol. Chem., 273 , 23381-23387 (1998).



Structure and Active Site of the BPO Subunit from *Cor. pilulifera*

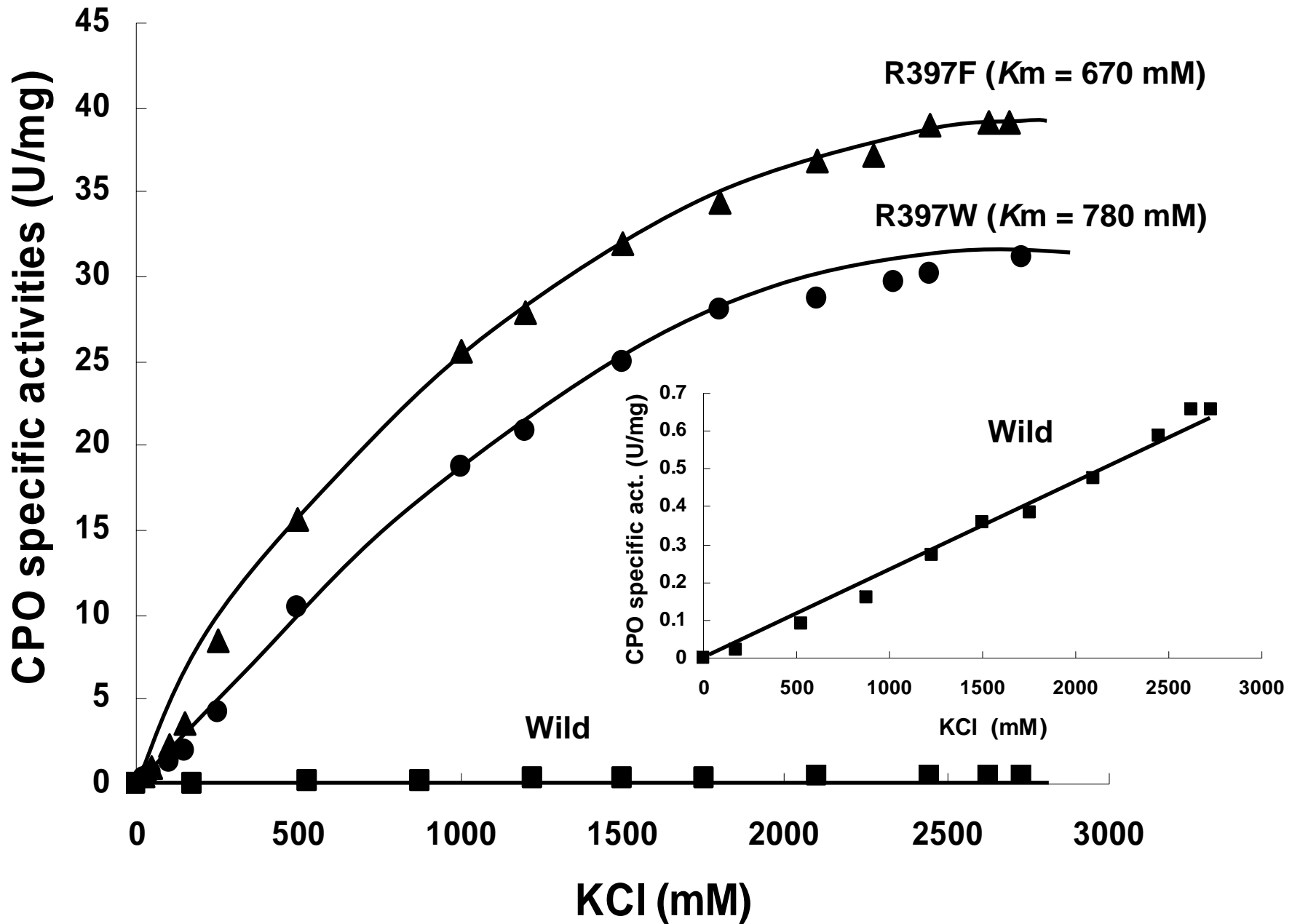


Activities of Mutant Enzymes in Cell-free Extracts

Comparison of Properties among Recombinant Wild Type BPO, Mutant BPOs (R397W and R397F)

	Wild	R397W	R397F
Molecular weight	680 kDa	680 kDa	680 kDa
Subunit weight	64 kDa × 12	64 kDa × 12	64 kDa × 12
Optimum temp.	65°C	65°C	65°C
Optimum pH	6	6	6
Heat stability (20 min)	85°C (90%)	85°C (90%)	85°C (90%)
pH stability	5-13	3-13	5-13
Specific activity	432 U/mg (BPO) 0.65 U/mg (CPO)	469 U/mg (BPO) 25.1 U/mg (CPO)	459 U/mg (BPO) 32.0 U/mg (CPO)
<i>K_m</i> value	0.12 mM (H ₂ O ₂) 8.4 mM (KBr) N.D* (KCl)	0.13 mM (H ₂ O ₂) 7.1 mM (KBr) 780 mM (KCl)	0.081 mM (H ₂ O ₂) 4.25 mM (KBr) 670 mM (KCl)
Inhibitor	Cu ²⁺ , 8-quinolinol Iodoacetate	Cu ²⁺ , 8-quinolinol Iodoacetate Sodium azide	Cu ²⁺ , 8-quinolinol Iodoacetate Sodium azide

*Not determined



Measuring K_m Values with KCl Concentrations

1. ピリヒバ由来ハロペルオキシダーゼ

(I) 酵素の精製、構造解析

(II) 安定性とカルシウム

(III) 変異酵素のハライドに対する活性

2. 緑藻(アワビモ、ミル)由来ハロペルオキシダーゼ

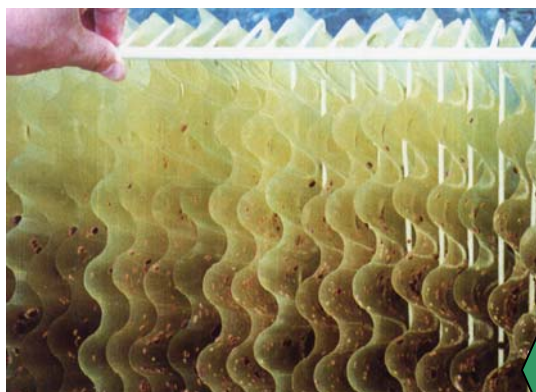
**アワビモ (*Ulvella lens*) 由来ハロペルオキシダーゼ**

Occurrence of bromoperoxidase in the marine green macro-alga, *ulvella lens*, and emission of volatile brominated methane by the enzyme

Takashi Ohshiro^a, Satoru Nakano^b, Yoshinori Takahashi^b, Minoru Suzuki^b,
Yoshikazu Izumi^{b,*}

^aDepartment of Biotechnology, Tottori University, Tottori 680-8552, Japan

^bDivision of Material Science, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Japan



ウニの人工養殖において、幼生の着底、
変態誘起にアワビモが用いられている

ハロメタンが関与??

アワビモ由来ハロペルオキシダーゼ
を精製するため、アクリル板上に
付着したアワビモを、北海道栽培漁業
振興公社より送っていただいた。

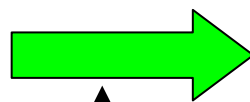
活性を確認、金属依存型酵素(特にコバ
ルト)、オキザロ酢酸からブロモメタンの
生成を確認



海藻ミル (*Codium fragile*) の無細胞抽出液調製



藻体12.1 kg

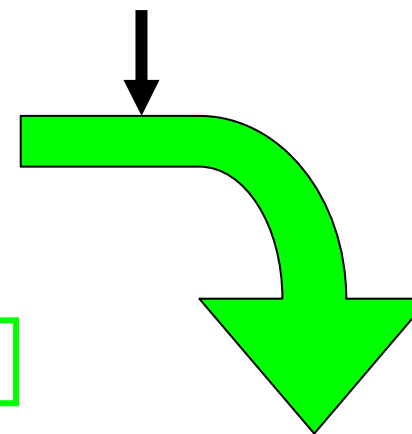


液体窒素で
凍結



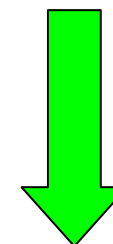
ミキサーで破碎

10 mM Tris-HCl buffer (pH 7.0)

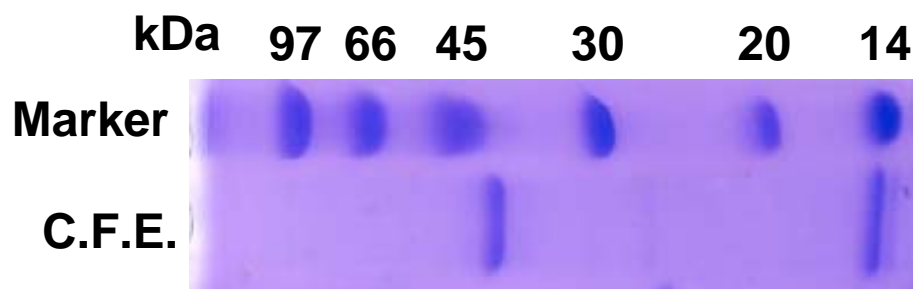


遠心分離

(8,000 rpm、60 min、4°C)



無細胞抽出液

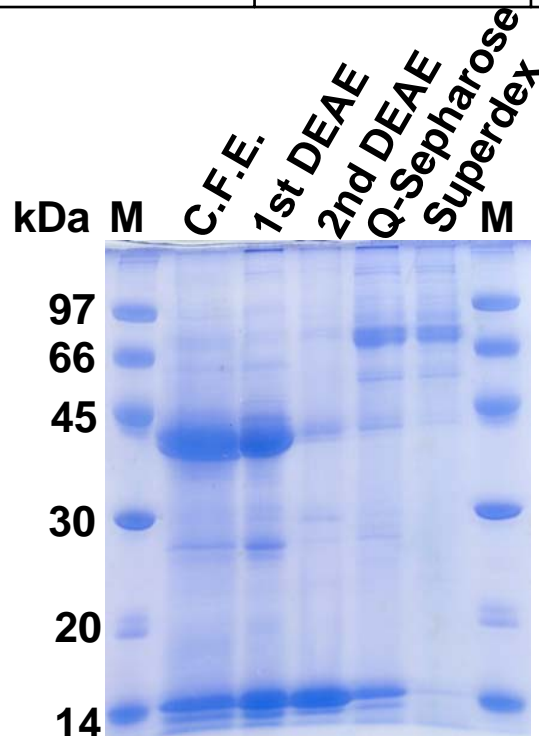


総タンパク質量 1,217 mg

海藻ミル由来ハロペルオキシダーゼの精製

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
ミルC.F.E.	768	212	0.277	1	100
1st DEAE	238	32	0.136	0.5	15
2nd DEAE	94	18.8	0.200	0.7	8.9
Q-Sepharose	3.5	4.3	1.24	4.5	2.0
Superdex	0.18	0.64	3.56	12.9	0.3

ミル藻体 10 kg (湿重量) から酵素精製



- ・金属 (Ca、Mn) で活性増大
- ・ピリヒバ由来酵素に比べ、熱安定性は低い (60°C、20 min 処理で失活)

海藻ミル由来ハロペルオキシダーゼの諸性質

	至適 pH	K_m [H_2O_2] (mM)	K_m [KBr] (mM)	分子量 (kDa)
<i>C. fragile</i> (緑藻)	6.0	0.015	35	72 ¹⁾ , 417 ²⁾
<i>P. capitatus</i> (緑藻)	4.0	0.125	30	52 ²⁾
<i>P. lamourouxii</i> (緑藻)	4.0	0.125	15	48 ²⁾
<i>C. pilulifera</i> (紅藻)	6.0	0.092	11	67 ¹⁾ , 790 ²⁾
<i>A. nodosum</i> (褐藻)	6.0	0.034	13	40 ¹⁾ , 90 ²⁾

1) subunit 2) native

本酵素は他の緑藻由来ハロペルオキシダーゼと
異なる特性を有する！

ハロゲン化酵素の現状と今後の課題

- ピリヒバ由来の酵素は、他のハロペルオキシダーゼよりも高い熱安定性、溶媒耐性、比活性
- クロロ活性が低い

さらに機能改変された変異酵素の構築



実際のハロゲン化反応への適用