

**Biochemical studies and applications of microbial
cytochrome P450 monooxygenases and
molybdenum-containing oxidoreductases**

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ABBREVIATIONS

4-AA	4-aminoantipyrine
AMP	adenosine monophosphate
BMAL	2-methyl-3-(phenylmethoxy)-4 <i>H</i> -pyran-4-one
BMAL-CHO	3-benzyloxy-4-oxo-4 <i>H</i> -pyran-2-carboxaldehyde
BMAL-COOH	3-benzyloxy-4-oxo-4 <i>H</i> -pyran-2-carboxylic acid
BMAL-OH	2-(hydroxymethyl)-3-(phenylmethoxy)-4 <i>H</i> -pyran-4-one
CFEs	cell-free extracts
CYP	cytochrome P450 monooxygenase
DCIP	2,6-dichlorophenolindophenol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ESI-MS	electrospray ionization mass spectrometry
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
FPLC	fast protein liquid chromatography
FDR	ferredoxin reductase
FDX	ferredoxin
FMN	flavin mononucleotide
GMP	guanosine monophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IMP	inosine monophosphate
IPTG	isopropyl- β -thiogalactopyranoside
KPB	potassium phosphate buffer
LB	Luria-Bertani
LC-MS	liquid chromatography mass spectrometry
MTT	3-[4,5-dimethylthiazol- 2-yl]-2,5-diphenyltetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PES	phenazine ethyl sulfate
PMS	phenazine methosulfate
rDNA	ribosomal DNA
SDS	sodium dodecyl sulfate
TER	transepithelial electrical resistance
TOOS	N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline
UV	ultraviolet
XDH	xanthine dehydrogenase
XOD	xanthine oxidase
XOR	xanthine oxidoreductase

GENERAL INTRODUCTION

Microorganisms are used in various industrial fields. For medical applications, microbial secondary metabolites, enzymes, and live cells are mainly used. As microbial secondary metabolites, their complicated chemical structures are very attractive, and they are used for antibiotics, antivirals, anticancers, and so on. As microbial enzymes, they are used for green chemistry, which has lower risk of environmental pollution, and for asymmetric synthesis because of their high regio-specificity. Recent remarkable progress of molecular biology contributes the bioprocess development. Moreover, microbial enzymes are used themselves for medicines such as digestives and for diagnostic products. As microbial live cells, valuable enterobacteria start to be used for disease prevention and management. From the recent development of microbiome researches, it is revealed that human microflora dysbiosis are related to various diseases such as immune disorder and psychiatric disorder.

In this study, for microbial bioprocess development, the author investigated the enzymatic synthesis of maltol derivative, which is valuable for pharmaceutical intermediate (chapter I, II). Maltol (3-hydroxy-2-methyl-4-pyrone) derivatives are used in the food, beverage, cosmetic and pharmaceutical industries due to their metal chelating abilities, and in particular, the hydroxypyrrone region of maltol is known to have potent transition metal chelating capability (1). Maltol has also been used as a ligand for vanadyl ions in insulin enhancing agents for treatment of diabetes mellitus, and a bis-maltol-vanadium complex showed improved pharmacokinetic parameters when compared to vanadium alone (2). Maltol derivatives have tyrosinase inhibitory activity due to their ability to chelate copper at the active site of the enzyme. Tyrosinase is involved in melanogenesis, enzymatic browning of fruits and vegetables, parasite encapsulation and sclerotization in insects, hence maltol derivatives are used as skin-whiteners, food preservative, and insecticides (3, 4). Metalloproteinase inhibitor activity is also found in maltol derivatives, and is enhanced by extending their 2-methyl side chain, which non-covalently interacts with specific subsites neighboring the active site of the enzyme (5,6). Although it is difficult to activate the methyl group by chemical synthesis, some biocatalysts are known to oxidize this type of non-active methyl group. Cytochrome P450 enzymes (P450s) are heme b-containing monooxygenases which are defined as a distinct class of hemoproteins (7). Because P450s catalyze regio- and stereo-specific oxidation of non-activated hydrocarbons under mild conditions, they have been used for biotechnological applications (8,9,10). Their reaction mechanism and structure-function relationship are well documented. To facilitate the activity of P450s, one or more redox partner proteins are required for sequential delivery of two electrons (11), and there are many reports of molecular engineering approaches to improve the catalytic activity and substrate selectivity of P450s (12,13). The 2-carboxylic acid group of maltol derivative could be a chemical modifying point for expanding the functional diversity. The author attempted to build a one-pot conversion system of maltol derivative to their 2-carboxylic acid by combination of multistep oxidation activity of P450 and aldehyde oxidoreductase.

For the application of microbial enzymes and probiotics, the author investigated the enzyme and microorganism used for hyperuricemia prevention by decreasing the amount of purines in human body (chapter III, IV). Eating foods rich in purines contributes to the total uric acid pool. Elevated level of serum uric acid causes hyperuricemia, a risk factor for acute gouty arthritis, chronic tophaceous gout, renal stones, and possibly cardiovascular events and mortality. Purines in foods are degraded into mononucleotide by ribonuclease, deoxyribonuclease, and polynuclease in intestine. All nucleotide are digested by nucleotidase to corresponding nucleoside and inorganic phosphate. The research *in vitro* with surviving everted intestinal sacs of rat showed that AMP, GMP, adenosine and guanine were easily degraded (14). Another study using rat intestine demonstrated that inosine, guanosine, hypoxanthine, xanthine, and guanine were converted to uric acid and absorbed from jejunum to serosal side as soon as their metabolism and absorption in jejunum (15, 16). Uric acid itself was also absorbed from jejunum to the serosal side of the intestinal sac by passive diffusion (17). Effect of oral purines on serum and urinary uric acid of normal, hyperuricemic and gouty humans was previously reported and oral AMP, IMP, GMP, adenine, and hypoxanthine elevated serum uric acid levels, while xanthine and guanine did not affect serum uric acid (18). Concerning about the purine level in food, wide variation were investigated in the absolute and/or relative levels of adenine, hypoxanthine, xanthine and guanine in food items ordinarily consumed in the human diet (19). Low purine diet is essential but traditional low-purine diets, once a mainstay of hyperuricemia management, are difficult for patients to adhere to because it is not easy to keep the nutritional balance of their diets. Additionally, over production and/or under excretion of uric acid are also causes of hyperuricemia. Against these symptoms, therapeutic drugs like promoter of uric acid excretion and inhibitor of uric acid production are developed. The former acts on kidney tubules and increases urinary excretion of uric acid, thus lowering blood uric acid levels. The latter is a xanthine oxidase inhibitor, thus reducing the production of uric acid. These are helpful in treating patients with hyperuricemia. However there are prospects of side-effects such as liver and/or kidney damage because of dosing these drugs. Considering these facts, the author's group attempted to apply orally administered microorganisms for helping inosine and guanosine digestion making lower the elevated serum uric acid level derived from food in human intestine. As a result using hyperuricemia model rats experiments, *Lactobacillus fermentum* ONRIC b0185, *L. fermentum* ONRIC b0195, and *L. pentosus* ONRIC b0223 were selected as potential strains for lowering the elevated serum uric acid level (20). The analysis of purine metabolism of these three strains demonstrated that hypoxanthine was not degraded to xanthine, while that inosine was converted to hypoxanthine. The previous paper reported the rank of purines with respect to their hyperuricemic effect per unit of purine fed. According to this paper, xanthine and uric acid effected much less than hypoxanthine for the gouty subjects (18). The author attempted to propose a new prophylaxis for hyperuricemia applying enzymes derived from microorganisms, which decomposed hypoxanthine which is known to have potency of increasing serum uric acid level in purine bases. Through the screening, hypoxanthine degrading microorganisms were isolated, and the enzyme involved

in the metabolism was purified, cloned, and characterized. Moreover, the author established a novel evaluation system with Caco-2 cell which was focused on the purine base metabolizing or adsorbing activity of probiotic bacteria in order to get much more accurate estimation for lowering serum uric acid level.

Chapter I

Engineering of the cytochrome P450 monooxygenase system for benzyl maltol hydroxylation

In chapter I, the author focused on benzyl maltol (BMAL), a maltol derivative, and examined the development of a 2-methyl side chain hydroxylation biocatalyst for BMAL. The 2-hydroxymethyl group in the reaction product could be a chemical modifying point for expanding the functional diversity of maltol derivatives. P450nov, FDXnov, and FDRnov were identified as the enzymes responsible for BMAL hydroxylation in *Novosphingobium* sp. SB32149, and enhanced their catalytic activity using a directed evolution technique. Moreover, several sets of redox partner proteins were tested for their compatibility with P450nov for efficient electron transport during BMAL hydroxylation (Fig. 1-1).

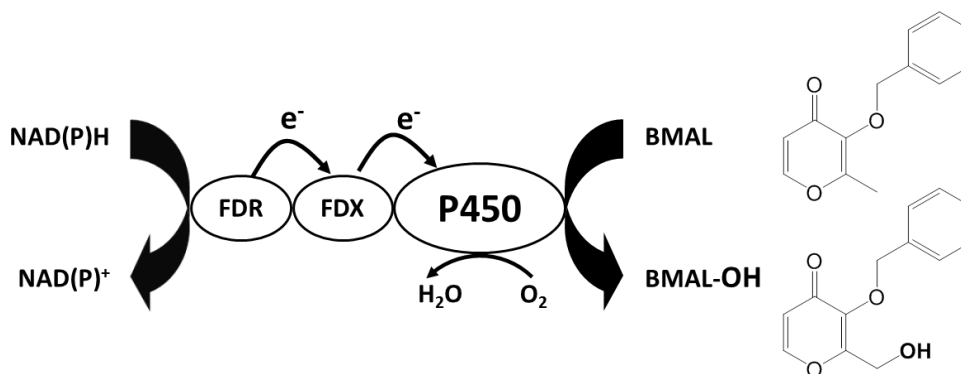


Fig. 1-1 Diagram of BMAL hydroxylation to BMAL-OH catalyzed by P450

Electrons are transferred from NAD(P)H via FDR (ferredoxin reductase) and FDX (ferredoxin) to P450, which activates molecular oxygen and incorporates one oxygen atom into the BMAL substrate by means of hydroxylation, while the other oxygen is reduced to water.

Materials and methods

Materials

An *E. coli* library expressing 230 types of bacterial P450s (21), the P450-expressing vectors pCbCa (22) and pAbAc (23), and *Escherichia coli* BL21(DE3) lacking the gene encoding the multidrug efflux pump *tolC* (24) were provided from MicroBiopharm Japan Co., Ltd. (Tokyo, Japan). 2-methyl-3-(phenylmethoxy)-4H-pyran-4-one (BMAL) and 2-(hydroxymethyl)-3-(phenylmethoxy)-4H-pyran-4-one (BMAL-OH) were synthesized as previously described (25, 26). Soil samples were collected in Kinki region of Japan. Primers used in this study are listed in Table 1-1.

Table 1-1 Primer combinations used in the present work

Primer name	Primer sequence
deg_P450_F	AMCGGCGGNCACTGGATCGCVAC
deg_P450_R	GAAGCYGAGGAAGTTSACVACVGTATCG
inv_P450_F	ATCACACAGATGGTCAACACC
inv_P450_R	AGAGGAAGGTCTGCCAGAGC
nest_P450_F	TGGTCAACACCGAGATCAACG
nest_P450_R	AGAGCCATGAACACGTGAACG
NdeI_F	AGACATATGATGAATCATCTCACGAC
SpeI_R	ACTAGTTCAGCGATCGACGGACCAG
ovlp_NdeI_F	AGACATATGATGAATCATCTCACGAC
ovlp_link_R	AGGATCTTCAGCGATCGACGGACCAG
ovlp_link_F	ATCGCTGAAGATCCTGAACTGAGTG
ovlp_BglII_R	ACCAGATCTGCATCGAACATCGAACC
ovlp_NcoI_F	ATGTCCATGGCGACCATAACCGTAATCTCG
ovlp_link_R2	GATCCAGCTGACGTGTCAGTCTTCGGGCGC
ovlp_link_F2	CGCGCCCGAAGACTGACACGTCAGCTGGATC
camA_F	GTCCGTCGATCGCTGAACTAGTCGGGAGTGCG
camA_BlnI_R	GACTCCTAGGTCAGGCACTACTCAGTTCAGC
rdm1_NdeI_F	CCTGAGTTTCAGATGTAACACGTCAGC
rdm1_BglII_R	ACCCCGATGATCACCAGATCTGC
L188P_F	GGAAGACGTGCCGCCTCTAAGTGTATTCG
L188P_R	CGAATACACTTAGAGGCGGCACGTCTTCC
inv_FDX_F	GTGGAGGGCCGCACAG
inv_FDX_R	ATGTGCTGTGGGAAATG
deg_FDR_F	CCGCCGCTGTCCAAGGAGTAC
deg_FDR_R	GTACTGGTTCGACCAGAACC
inv_FDR_F	TGCTGGCGCGCGTCCGCGG
inv_FDR_R	AGGGCTTCGAGCAGGACCAC
FDRnov_SpeI_F	TGAACTAGTCGGGAGTGCGTTATATGAAAAACCAGACGTCGTC
FDRnov_BlnI_R	GCAGCCTAGGTTATCACACCAGTTCCTTGAGCGG
rdm2_NcoI_F	ATGTCCATGGCGACCATAACCGTAATCTCG
rdm2_SpeI_R	ACTAGTTCAGCGATCGACGGACCAG

Screening of bacterial P450 library for BMAL hydroxylating activity

The bacterial P450-expressing *E. coli* cells were cultivated at 30 °C with shaking (300 rpm) in P450 seed medium (1 mL in a 17 mm X 100 mm test tube) composed of 11.2 g/L 5 × M9 minimal salts (Becton Dickinson, NJ, USA), 10 g/L casamino acids, 4 g/L D-glucose, 0.02 g/L thymine, 0.02 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L carbenicillin, and 28 µg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. After cultivation for 15 h, 1% of seed culture was added to P450 expression medium (0.5 mL in a 17 mm X 100 mm test tube) composed of 11.2 g/L 5 × M9 minimal salts, 10 g/L casamino acids, 12.6 g/L glycerol, 0.02 g/L thymine, 0.08 g/L 5-aminolevulinic acid, 0.1 g/L carbenicillin, 20 mL/L Overnight Express Autoinduction System (O.E.A)-Solution I (Merck Millipore, Darmstadt, Germany), 50 mL/L O.E.A-Solution II, 1 mL/L O.E.A-Solution III, and 28 µg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. After cultivation for 24 h at 25 °C with shaking (300 rpm), a 0.5 mL aliquot of P450-expressing cells was centrifuged and the cell pellet was resuspended in 100 µL of resting cell reaction mixture, consisting of 6.8 g/L Na_2HPO_4 , 3 g/L NaH_2PO_4 , 25 g/L glycerol, 0.05 g/L carbenicillin, 0.02 g/L IPTG, and 14 g/L hydroxypropyl β -cyclodextrin, in a 96-well assay block (0.5 mL; Corning, NY, USA). BMAL dissolved in DMSO was added at a final concentration of 100 mg/L and the mixture was shaken at 1,000 rpm using a 96-well plate shaker (TAITEC, Saitama, Japan) at 28 °C. After 24 h of reaction, the mixture was centrifuged and the supernatant was mixed with an equal volume of ethanol. BMAL-OH in the solution was analyzed by high performance liquid chromatography (HPLC).

Enrichment for camphor-assimilating microorganisms

Approximately 1 mg of each soil sample was suspended in 1 mL of M9 minimal medium in 96-well assay blocks (1 mL; Corning). The suspensions were sonicated and centrifuged, and then the supernatants were added to 0.5 mL of enrichment medium containing 11.28 g/L 5 × M9 minimal salts, 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.7 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mL/L metal mixture (0.1 g/L H_3BO_3 , 0.5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L KI, 2 g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/L H_2SO_4), and 200 mg/L camphor as a sole carbon source, pH 7.0, in 96-well assay blocks (1 mL; Corning). Enrichment cultivation was carried out in a 96-well plate shaker at 30 °C, and 10% of the volume of the culture was transferred to fresh enrichment medium every 2 days. After passaging twice, the resultant culture was centrifuged and the supernatant was mixed with an equal volume of ethanol. Camphor metabolites in the solution were analyzed by HPLC. The camphor assimilating microorganisms were stored in Nutrient Agar Broth (Becton Dickinson).

Screening of BMAL-hydroxylating microorganisms

The camphor-assimilating microorganisms were inoculated into BMAL medium containing 11.28 g/L 5 × M9 minimal salts, 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.7 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mL/L of metal mixture as described above, 200 mg/L camphor, and 100 mg/L BMAL, and were cultivated in a 96-well plate shaker at 30 °C. After cultivation for 2 days, the culture was centrifuged and the supernatant was mixed

with an equal volume of ethanol. BMAL-OH in the solution was analyzed by HPLC. Single colony isolation of BMAL-OH-producing strains was carried out on Nutrient Agar Broth. Microbial identification was carried out by 16S rDNA sequence analysis using a MicroSeq Full Gene 16S rDNA Bacterial Identification kit (Applied Biosystems, CA, USA).

Cloning of the CYP101-encoding gene from *Novosphingobium* sp. SB32149

Genomic DNA from *Novosphingobium* sp. SB32149 was isolated using a QIAGEN Blood & Cell Culture DNA Kit (QIAGEN, CA, USA), and was used as a PCR template with degenerate primers (deg_P450_F and deg_P450_R) designed from sequence regions highly conserved within the CYP101 family. PCR was performed with PrimeSTAR HS DNA polymerase (TakaraBio, Kyoto, Japan) according to the following protocol: 98 °C for 2 min; 30 cycles of 98 °C for 10 s, 60 °C for 5 s, and 72 °C for 1 min; and 72 °C for 2 min. The PCR product was cloned into a pCR-BluntII-TOPO vector using a Zero Blunt TOPO cloning kit (Thermo Fisher Scientific, MA, USA). The inserted gene fragment was sequenced on a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA). For preparation of the DNA template used for inverse PCR, the genomic DNA of *Novosphingobium* sp. SB32149 was digested with restriction enzymes (*EcoRI*, *HindIII*, *PstI*, *SpeI*, *KpnI*, *NdeI* and *XhoI*) and self-ligated with a DNA Ligation Kit (Merck Millipore, Darmstadt, Germany). The circularized DNA was used as a template for PCR with inverse primers (inv_P450_F and inv_P450_R) designed from the sequence of the gene fragment obtained as above. The resulting PCR product was used as a template for PCR with nested PCR primers (nest_P450_F and nest_P450_R). PCR using both inverse and nested primers was performed using the following conditions: 98 °C for 2 min; 25 cycles of 98 °C for 10 s, 55 °C for 5 s, 72 °C for 3 min; and 72 °C for 2 min. The PCR products amplified from the genomic DNA treated with *EcoRI*, *HindIII* and *PstI*, were cloned into a pCR-BluntII-TOPO vector and sequenced. The sequences were combined and the full-length sequence of the gene was obtained. The resulting gene product was named P450nov (GenBank accession number: LC214353).

Cloning of ferredoxin and ferredoxin reductase from *Novosphingobium* sp. SB32149

The ferredoxin gene located near P450nov in *Novosphingobium* sp. SB32149 genomic DNA was identified by the genome walking method. Inverse PCR was performed with inv_FDX_F and inv_FDX_R primers using *SalI* treated and circularized genomic DNA as template. Partial ferredoxin reductase of this strain was amplified by degenerate PCR using deg_FDR_F and deg_FDR_R primers designed from a conserved amino acid region of ArR from *Novosphingobium aromaticivorans* DSM12444, CamA from *Pseudomonas putida* ATCC17453, and PuR from *Rhodopseudomonas palustris* CGA009. The full length ferredoxin reductase gene was determined by inverse PCR using inv_FDR_F and inv_FDR_R primers with *NcoI* treated and circularized genomic DNA as template. The gene products of ferredoxin and ferredoxin reductase in SB32149 were named FDXnov and FDRnov (GenBank

accession number: LC214354, LC214355), respectively.

Construction of P450nov expression vectors

Using the plasmids, primers, and restriction enzymes described in Table 1-2, expression vectors of P450nov-related genes, with varying combinations of electron transporters, were constructed. The vector nov_CbCa, containing the P450nov gene amplified from the SB32149 genome, was constructed with the pCbCa vector as a backbone. The vector nov_AbAc was constructed with pAbAc as a backbone vector and the P450nov-partial aciC gene amplified by overlap extension PCR using nov_CbCa and pAbAc. The vector nov_FxAC was also constructed using overlap extension PCR with the FDX gene amplified from the S32149 genome and the P450nov-partial aciC gene from nov_AbAc, with pAbAc as a backbone vector. The vector nov_FxCa was constructed by replacing the aciC gene of nov_FxAC with the camA gene amplified from pCbCa. Similarly, the vector nov_FxFr was constructed by replacing the camA gene of nov_FxCa with the FDRnov gene amplified from the SB32149 genome.

Table 1-2 Plasmids, primers, and ligation sites used for construction of expression vectors and mutant library

Plasmid No.	Constitution of plasmid			Construction of plasmid				Plasmid (Transformant) Name
	P450nov	FDX	FDR	Primers	Template (Insert)	Vector arm	Ligation sites	
1	-	camB	camA	-	-	-	-	pCbCa
2	-	aciB	aciC	-	-	-	-	pAbAc
3	wild type	camB	camA	NdeI_F, SpeI_R	SB32149	1	NdeI, SpeI	nov_CbCa
4	wild type	aciB	aciC	a)ovlp_NdeI_F, ovlp_link_R b)ovlp_link_F, ovlp_BglII_R c)ovlp_NdeI_F, ovlp_BglII_R	a)3 b)2 c)a+b	2	NdeI, BglII	nov_AbAc
5	wild type	FDXnov	aciC	a)ovlp_NcoI_F, ovlp_link_R2 b)ovlp_link_F2, ovlp_BglII_R c)ovlp_NcoI_F, ovlp_BglII_R	a)SB32149 b)2 c)a+b	2	NcoI, BglII	nov_FxAc
6	wild type	FDXnov	camA	camA_F, camA_BlnI_R	1	5	SpeI, BlnI	nov_FxCa
7	wild type	FDXnov	FDRnov	FDRnov_SpeI_F, FDRnov_BlnI_R	SB32149	6	SpeI, BlnI	nov_FxFr
8	E103K	aciB	aciC	rdm1_NdeI_F, rdm1_BglII_R	4	4	NdeI, BglII	-
9	L188P	aciB	aciC	rdm1_NdeI_F, rdm1_BglII_R	4	4	NdeI, BglII	-
10	F218L	aciB	aciC	rdm1_NdeI_F, rdm1_BglII_R	4	4	NdeI, BglII	-
11	A381V	aciB	aciC	rdm1_NdeI_F, rdm1_BglII_R	4	4	NdeI, BglII	-
12	E103K	FDXnov	camA	-	8	6	NdeI, SpeI	-
13	L188P	FDXnov	camA	-	9	6	NdeI, SpeI	-
14	F218L	FDXnov	camA	-	10	6	NdeI, SpeI	-
15	A381V	FDXnov	camA	-	11	6	NdeI, SpeI	-

16	L188P,F218L	FDXnov	camA	L188P_F, L188P_R	14	-	-	novPL_FxCa
17	E103K,F218L	FDXnov	camA	-	10	12	BamHI, SpeI	-
18	F218L,A381V	FDXnov	camA	-	10	15	XbaI, AatII	-
19	E103K,L188P	FDXnov	camA	-	9	12	BamHI, SpeI	-
20	L188P,A381V	FDXnov	camA	-	9	15	XbaI, AatII	-
21	E103K,A381V	FDXnov	camA	-	11	12	BamHI, SpeI	-
22	E103K,L188P, F218L	FDXnov	camA	-	12	16	BamHI, SpeI	-
23	L188P,F218L, A381V	FDXnov	camA	-	16	15	XbaI, AatII	-
24	E103K,F218L, A381V	FDXnov	camA	-	17	15	BamHI, SpeI	-
25	E103K,L188P, A381V	FDXnov	camA	-	20	12	BamHI, SpeI	-
26	E103K,L188P, F218L,A381V	FDXnov	camA	-	23	12	BamHI, SpeI	-
27	L188P,F218L	FDXnov	FDRnov	-	16	7	NdeI, SpeI	novPL_FxFr
28	L188P,F218L, L237M	FDXnov	FDRnov	rdm2_NcoI_F, rdm2_SpeI_R	27	27	NcoI, SpeI	novPLM_FxFr
29	L188P,F218L	FDXnov (A10T)	FDRnov	rdm2_NcoI_F, rdm2_SpeI_R	27	27	NcoI, SpeI	novPL_FxTFR
30	L188P,F218L, L237M	FDXnov (A10T)	FDRnov	-	28	29	NdeI, Spe I	novPLM_FxTFR

Expression of P450nov and redox partner proteins in *E. coli* and the conversion of BMAL

E. coli BL21(DE3) (*tolC::Tn10*) containing P450nov and redox partner protein genes was cultivated at 30 °C with shaking (1,200 rpm) in 200 µL of the P450 seed medium in 96-well assay blocks (0.5 ml; Corning). After 15 h of cultivation, 4 µL of seed culture was added to 400 µL of the P450 expression medium in 96-well assay blocks (0.5 ml; Corning). After cultivation for 24 h at 25 °C with shaking (1200 rpm), the culture was used for BMAL conversion as follows. For the growing cell reaction, the P450 expression culture was added to BMAL in DMSO together with 1% glycerol, and 10 mM hydroxypropyl β -cyclodextrin was added for growth promotion or high substrate solubility. For the resting cell reaction, centrifuged cells from 400 µL of culture were added to equal or half volume of the resting cell reaction mixture (1 X or 2 X resting cell reaction). In each reaction, BMAL in DMSO was added and the mixture was shaken (1200 rpm) at 28 °C. When evaluating the stability of enzymes, cell suspension was pretreated at 45 °C for 45 min before substrate addition or reaction temperature was raised at 37 °C. Cell growth was evaluated by measuring OD600 with a UV-visible spectrophotometer (Ultrospec 2000; GE healthcare, Little Chalfont, UK). Functional CYP concentration in the cells was quantitated by measuring the CO difference spectra as described previously (27) with a UV-visible spectrophotometer (U-3310; Hitachi, Tokyo, Japan).

Gene mutations of P450nov and FDXnov

Introduction of random mutations (0-4.5 mutations/kbp) was performed using a Genemorph II Random Mutagenesis Kit according to the manufacturer's instructions (Agilent, CA, USA). In the first round of random mutation, the P450nov gene of nov_AbAc was amplified with rdm1_NdeI_F and rdm1_BglII_R primers. PCR was performed with Mutazyme II DNA polymerase using the following program: 95 °C for 2 min; 28 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 126 s; and 72 °C for 10 min. The PCR product was digested with *NdeI* and *BglII*, and ligated into the pAbAc vector. In the second round of random mutation, the gene fragment encoding P450nov L188P/F218L and FDXnov of novPL_FxFr was amplified with rdm2_NcoI_F and rdm2_SpeI_R primers. The PCR product was digested and ligated into the *NcoI* and *SpeI* site of novPL_FxFr. The L188P/F218L double mutant was constructed using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent). Using plasmid 12 (F218L) as a template, the L188P mutation was introduced with L188P_F and L188P_R primers. PCR was performed with *PfuUltra* High Fidelity DNA polymerase using the following program: 95°C for 1 min; 18 cycles of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 8 min; and 68 °C for 7 min. The L188P/F218L/L237M triple mutant and A10T mutant of FDXnov were constructed by second round random mutagenesis. Using plasmids and restriction enzymes described in Table S2, mutation and electron transporter combinations were constructed by ligation. The resultant plasmids were transformed into *E. coli* BL21(DE3) (*tolC::Tn10*) and used for the BMAL conversion reaction as described above.

BMAL-OH production in jar fermenter

NovPLM_FxTFR-containing *E. coli* was inoculated into a test tube (17 mm X 100 mm) containing 1 ml of P450 seed medium and incubated at 30 °C with shaking at 300 rpm for 16 h. One milliliter of the seed broth was transferred into 100 ml of P450 expression medium in a 250 ml Bio Jr.8 jar fermenter (ABLE, Tokyo, Japan), and incubated at 25 °C with 600 rpm agitation and 1vvm aeration with pH maintained at 6.6 for 24 h. After 24 h, 4 g/L BMAL as substrate, 2 g/L glucose as a carbon source, and 10 ml of 100 mM hydroxylpropyl- β -cyclodextrin as an inclusion compound were added, and the culture was incubated at 28 °C. After 4, 9, and 24 h of cultivation, 1.5, 1.5, and 1.0 g/L BMAL and 5, 10, and 10 g/L glucose were added to the culture, respectively.

Results

CYP101 screening from soil microorganisms

A library of recombinant *E. coli* strains expressing various bacterial P450s (14) was screened for enzymes possessing hydroxylation activity towards BMAL. An *E. coli* strain expressing P450cam was found to produce 67.4 mg/L BMAL-OH from 100 mg/L BMAL after 24 h. Since P450cam is a member of the CYP101 family of enzymes known to have camphor-oxidizing ability (28), novel BMAL-hydroxylating enzymes were searched from camphor-assimilating microorganisms. A total of 2,925 soil microorganisms were isolated from the enrichment medium using camphor as a sole carbon source. Out of these, 437 strains metabolized camphor, 3 of which converted BMAL to BMAL-OH. According to the 16S rDNA sequence analysis, these BMAL-OH producing strains were identified as *Novosphingobium* sp. The strain with the highest activity, *Novosphingobium* sp. SB32149 (NBRC 112717), produced 5.8 mg/L BMAL-OH from 100 mg/L BMAL after 24 h, and was chosen for further investigation.

Cloning of genes encoding proteins involved in BMAL hydroxylation in *Novosphingobium* sp. SB32149

A P450 gene belonging to the CYP101 family was cloned from *Novosphingobium* sp. SB32149. This enzyme, P450nov, showed approximately 83% overall amino acid sequence homology with CYP101D2 of *N. aromaticivorans* DSM12444 (GenBank accession number: 499764401). CYP101 family enzymes require the redox partner proteins ferredoxin (FDX) and ferredoxin reductase (FDR) to transfer electrons from NAD(P)H into their heme center. Thus, FDX and FDR were also cloned from *Novosphingobium* sp. SB32149. The FDX gene of SB32149, FDXnov, was located at the 42 bp 3'-downstream region of the P450nov gene and showed approximately 72% overall amino acid sequence homology with FDX of *N. aromaticivorans* DSM12444 (GenBank accession number: ABD25920.1). The FDR gene of SB32149, FDRnov, showed approximately 74% overall amino acid sequence homology

with the FAD-dependent pyridine nucleotide-disulfide oxidoreductase of *N. aromaticivorans* DSM12444 (GenBank accession number: ABD24664.1).

Selection of electron transport system suitable for P450nov reaction in *E. coli*

The compatibility of P450 and redox partner proteins is important for the hydroxylating activity (29). For selecting the electron transport system for BMAL hydroxylation by P450nov, the following ferredoxin/ferredoxin reductase systems were examined: putidaredoxin (camB)/putidaredoxin reductase (camA), the electron transport system for P450cam in *Pseudomonas putida* ATCC17453 (30); aciB/aciC from *Acinetobacter* sp. OC4 (23); FDXnov/FDRnov, isolated in this study from *Novosphingobium* sp. SB32149; FDXnov/aciC; and FDXnov/camA. These electron transport systems were introduced into the P450nov-expressing *E. coli* strain (Table 1-2, Table 1-3), and the effect on BMAL hydroxylation was determined in the growing cell reaction. When cultivated with 100 mg/L BMAL for 28 h, BMAL-OH production was greater in the nov_AbAc recombinant *E. coli* strain (64.3 mg/L) compared to the nov_CbCa recombinant *E. coli* strain (9 mg/L) (Table 1-3). When cultivated with 1 g/L BMAL for 28 h, BMAL-OH production was greater in the nov_FxCa recombinant *E. coli* strain (0.683 g/L) than the nov_AbAc (0.031 g/L) and novFxAC (0.447 g/L) recombinant *E. coli* strains (Table 1-3). There was no difference in BMAL-OH production between the nov_FxCa (0.759 g/L) and nov_FxFr (0.742 g/L) recombinant *E. coli* strains when cultivated with 1.5 g/L BMAL for 28 h (Table 1-3).

Table 1-3 Effect of redox partner proteins on BMAL-OH production

Strain Name	P450nov	FDX	FDR	BMAL Addition (g/L)	BMAL-OH Production (g/L)
nov_CbCa	wild type	camB	camA	0.1	0.009
nov_AbAc	wild type	aciB	aciC	0.1	0.064
nov_AbAc	wild type	aciB	aciC	1.0	0.031
nov_FxAC	wild type	FDXnov	aciC	1.0	0.447
nov_FxCa	wild type	FDXnov	camA	1.0	0.683
nov_FxCa	wild type	FDXnov	camA	1.5	0.759
nov_FxFr	wild type	FDXnov	FDRnov	1.5	0.742
novPL_FxCa	L188P/F218L	FDXnov	camA	4.0	3.686
novPL_FxFr	L188P/F218L	FDXnov	FDRnov	4.0	3.618

Reactions were carried out in the growing cell reaction with P450nov wild type or L188P/F218L double mutant recombinant *E. coli*. Redox partners used in this reaction, amount of BMAL addition, and BMAL-OH production are indicated.

Directed evolution of P450nov to improve catalytic activity

To improve BMAL-hydroxylating activity, random mutagenesis was applied to the P450nov gene. Approximately 2,700 strains of *E. coli* expressing mutated P450nov were screened for BMAL-hydroxylating activity in the 2 X resting cell reaction at 25 °C with 100 µg/ml BMAL for 6 h. As shown in Table 1-4, 10 mutants had greater than twice the hydroxylating activity compared to wild type P450nov. The sequence analysis of these mutants revealed that 4 amino acid residues, E103, L188, F218 and A381, were hot spots for enhancing the activity of P450nov. Single mutations of E103, L188 and A381 enhanced BMAL-hydroxylating activity, and the F218 mutation was found in 3 out of the 10 mutants.

Next, the E103K, L188P, F218L, and A381V mutations were combined to improve the BMAL-hydroxylating activity of P450nov. Thirteen combination mutants were constructed and assayed in the growing cell reaction with 4 g/L BMAL for 32 h. Of the thirteen mutants constructed, a double mutant, L188P/F218L, had almost 12 times higher BMAL-hydroxylating activity when compared to the wild type (Table 1-5). There was no difference in BMAL-OH production when FDXnov/camA or FDXnov/FDRnov were used as the electron transport system in wild type P450 (Table 1-3). In order to increase P450 catalytic activity, the L188P/F218L mutant was used. Indeed, in cultivation with 4 g/L BMAL for 28 h, the BMAL conversion rates of the novPL_FxCa recombinant *E. coli* strain and the novPL_FxFr recombinant *E. coli* strain were both increased, but remained around the same level (3.686 g/L and 3.618 g/L, respectively) (Table 1-3). For evaluating the enzyme stabilities, the reaction temperature was increased from 28 °C to 37 °C in the 1 X resting cell reaction of P450nov (L188P/F218L) mutant-recombinant *E. coli* with addition of 4 g/L BMAL. In the 25 h reaction at 37 °C, the novPL_FxFr strain showed higher production of BMAL-OH (1.708 g/L) than novPL_FxCa (0.156 g/L) (Fig. 1-2).

Additional mutations were then carried out on P450nov L188P/F218L and FDXnov to improve catalytic activity and electron transport efficiency during BMAL hydroxylation. Because thermostable enzymes have been reported to have high stability and evolvability for improving catalytic activity (31,32), approximately 1,200 strains of mutated *E. coli* were pretreated at 45 °C for 45 min and then their hydroxylation activity was measured in the 2 X resting cell reaction with 2 g/L BMAL for 2 h. As a result, 34 mutants were obtained that had higher hydroxylating activity compared to novPL_FxFr. The BMAL conversion rate at 2 h and 24 h was measured in the 1 X resting cell reaction of these mutants to evaluate their initial velocity and activity persistence. Reactions were initiated at 37 °C with 4 g/L BMAL. Two mutants that had high activity at both 6 h and 22 h were selected and sequenced, and the L237M mutation of P450nov and A10T mutation of FDXnov were determined to be the effective mutations. The NovPLM_FxFr, novPL_FxTFR, and their combination of novPLM_FxTFR recombinant *E. coli* strains were compared to the parental novPL_FxFr strain in the growing cell reaction with 3 g/L BMAL at the start, and after 6 and 22 h of cultivation, 1.0 g/L BMAL was fed into the culture. BMAL-OH production

in all three mutants was increased (Table 1-6). Among them, novPLM_FxTFr showed the highest BMAL-OH production, 3.6 g/L.

Table 1-4 Evaluation of first random mutation library compared to wild type P450nov

Amino acid substitution	Relative activity
wild type	1.0
F218L,R428H	2.7
S11N, F218I	3.1
F70L, S190R	3.1
D128N, N202S, G234R	3.2
S11N, F218I, G258V	3.3
L188P	3.6
A381V	3.7
E103K	3.9
D128V, L188F	4.1
A177D, M306T, T388A	5.5

Relative activities of first random mutation library were presented, which were calculated based on BMAL-OH production (0.015 g/L) relative to wild type P450nov. Reactions were carried out in the resting cell reaction of aciB/aciC-containing *E. coli*. The 10 mutants that had greater than twice the BMAL-hydroxylating activity compared to wild type P450nov were sequenced.

Table 1-5 Evaluation of first combinatorial mutation library compared to wild type P450nov

Amino acid substitution	Relative activity
wild type	1.0
L188P,F218L,A381V	2.6
A381V	3.4
E103K,F218L	3.8
E103K,L188P	4.1
E103K,A381V	4.2
E103K,L188P,F218L	4.3
E103K,L188P,F218L,A381V	5.0
L188P	5.3
F218L,A381V	5.5
E103K	5.7
E103K,F218L,A381V	5.9
F218L	6.2
L188P,F218L	11.9

Relative activities of the first combinatorial mutation library are shown, calculated relative to the BMAL-OH production (0.104 g/L) of wild type P450nov. Reactions were carried out in the growing cells of FDXnov/camA-containing *E. coli*.

Table 1-6 Evaluation of second mutation library compared to the P450nov L188P/F218L mutant

Strain Name.	P450nov	FDXnov	FDRnov	BMAL-OH production (g/L)	
				2 h	24 h
novPL_FxFr	L188P/F218L	wild type	wild type	0.39	2.51
novPL_FxTFR	L188P/F218L	A10T	wild type	0.60	2.96
novPLM_FxFr	L188P/F218L/L237M	wild type	wild type	0.85	3.49
novPLM_FxTFR	L188P/F218L/L237M	A10T	wild type	0.94	3.60

Reactions were carried out in the growing cells with the P450nov L188P/F218L double mutant or L188P/F218L/L237M triple mutant recombinant *E. coli*. Redox partners used in the reaction and BMAL-OH production at 2 h and 26 h are indicated.

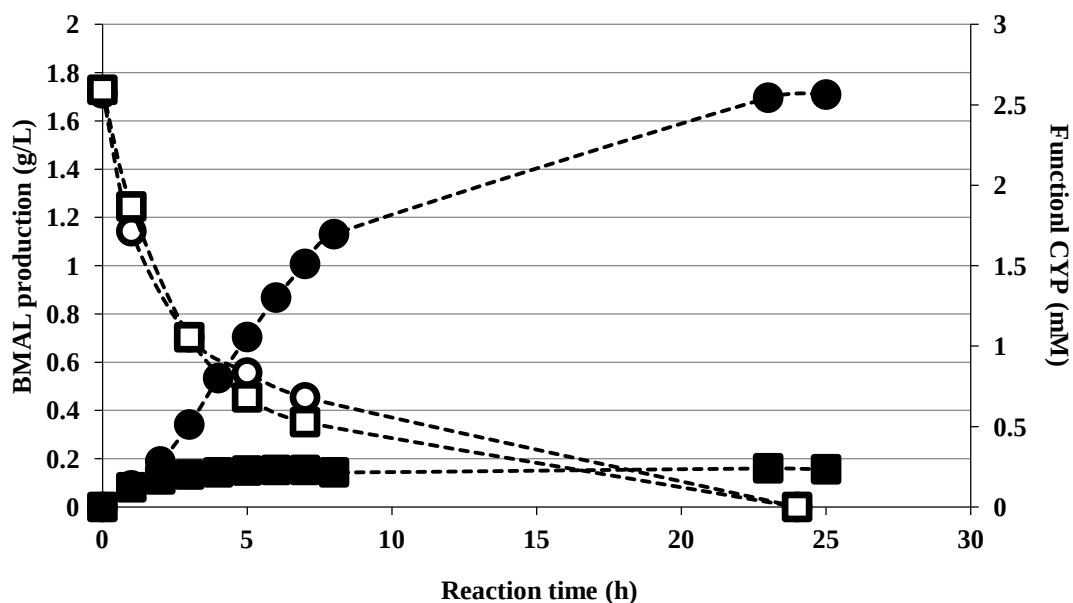


Fig. 1-2 Effect of redox partner proteins on BMAL-OH production and functional CYP concentration of P450nov L188P/F218L mutant

BMAL-OH production and functional CYP concentration are presented in a time-dependent manner. Reactions were carried out in the resting cell reaction with the P450nov L188P/F218L mutant. Functional CYP concentrations were measured by CO-difference spectrum. Closed symbols indicate BMAL production (g/L), open symbols indicate functional CYP concentration (mM). ferredoxin/ferredoxin reductase: $\circ$ ●; FDXnov/FDRnov, $\blacksquare$ □; FDXnov/camA

Preparative production of BMAL-OH in a jar fermenter

BMAL-OH was produced using novPLM_FxTFR under controlled conditions in a jar fermenter. The agitation rate, aeration rate, and culture pH were adjusted to 600rpm, 1vvm, and 6.6 respectively. The reaction was carried out in the growing cells at 28 °C with 4 g/L BMAL, and after 4, 9, and 24 h of cultivation, 1.5, 1.5, and 1.0 g/L BMAL and 5, 10, and 10 g/L glucose were added in the culture. Compared to the expression in the 96-well plate, the high aeration and pH control in the jar fermenter promoted the growth of P450nov-containing *E. coli* and the active P450nov concentration was increased (Table 1-7). Consequently, 5.2 g/L of BMAL-OH was produced from 8.0 g/L of BMAL in a 32 h reaction (Fig. 1-3).

Table 1-7 Comparison of the cell growth and the functional CYP concentration between two types of reactors

Reactor	OD (600 nm)	Functional CYP (μ M)
96-well plate	10.7	2.5
Jar fermenter	13.4	2.8

The novPLM_FxTFR recombinant *E. coli* was expressed in a 96-well plate or a jar fermenter at 25 °C for 24 h. After expression, the cell growth and the functional CYP concentration are shown.

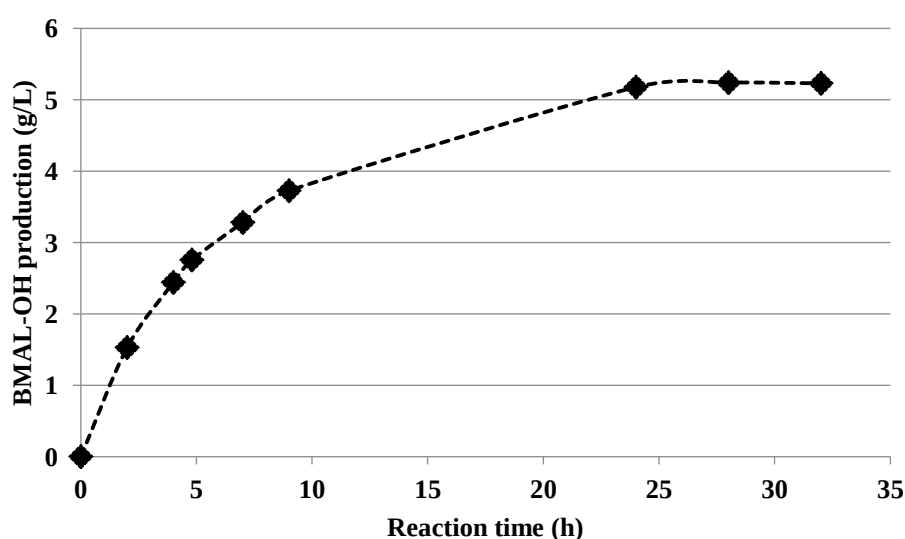


Fig. 1-3 Time course of BMAL-OH production in a jar fermenter reaction

BMAL-OH production in a jar fermenter is presented in a time-dependent manner. The reaction was carried out in the growing cell reaction with novPLM_FxTFR-recombinant *E. coli* under controlled conditions (600rpm, 1 vvm, pH6.6, 28 °C). Reaction was started with 4 g/L BMAL, and after 4, 9, and 24 h of cultivation, 1.5, 1.5, and 1.0 g/L BMAL and 5, 10, and 10 g/L glucose were added in the culture.

Discussion

In this study, the author reported the establishment of an *E. coli*-based biocatalyst for BMAL hydroxylation. Coupling of P450nov and its suitable redox partner proteins promoted efficient electron transportation during the hydroxylation of BMAL to BMAL-OH. Combination with camA/camB or aciB/aciC, which were derived from the other microorganisms, resulted in very low BMAL-OH production, less than 0.1 g/L. By replacing the ferredoxin with the native one, FDXnov, BMAL-OH production was increased to around 1.0 g/L. Moreover, combination with the native ferredoxin reductase,

FDRnov, enabled to produce more than 1.0 g/L BMAL-OH under high temperature condition. There was large difference in BMAL-OH production between FDRnov combination and camA combination, in spite of no difference in the active P450 concentrations (Fig. 1-2). It was indicating that the difference was caused by redox partners.

Furthermore, FDRnov was found to be more stable than camA. From these results, P450nov exerted the best catalytic activity when co-expressed with the native electron transporter FDXnov/FDRnov in terms of electron transportation efficiency and stability.

The amino acid substitution of L188P/F218L/L237M in P450nov and A10T in FDXnov resulted in increased BMAL-OH production. To determine the structural position of these amino acid residues in P450nov, homology modeling was performed using the structure of CYP101D2 (PDB No.: 3NV5) (33). It was speculated that 3 of the residues, L188, F218, and L237 are located at the substrate recognition site, thus these residues may interact directly with the BMAL substrate or regulate the conformation of the substrate binding pocket (34,35). Combination of multi efficient mutations around the substrate binding site could adjust the pocket to fit the substrate, BMAL, more suitably and improve the BMAL hydroxylating activity.

Finally, in a jar fermenter reaction under controlled culture conditions, 5.2 g/L of BMAL-OH was produced. It was approximately 1,000 times improvement of the productivity compared with that of original microorganism which was discovered from soil.

Summary

In chapter I, microbial enzymes were screened for hydroxylating activity towards the 2-methyl group in a maltol derivative, BMAL. *Novosphingobium* sp. SB32149 was found to have the ability to convert BMAL into BMAL-OH. The enzymes responsible, a cytochrome P450 monooxygenase (P450nov), a ferredoxin (FDXnov), and a ferredoxin reductase (FDRnov), were identified in the SB32149 strain. In the reaction with recombinant *E. coli* expressing P450nov, FDXnov, and FDRnov, BMAL-OH was successfully produced from BMAL. Moreover, using the directed evolution approach, four amino acid substitutions, L188P/F218L/L237M in P450nov and A10T in FDXnov, were found to enhance BMAL-OH production. Consequently, up to 5.2 g/L BMAL-OH was obtained from 8.0 g/L BMAL by bioconversion using a 250 mL jar fermenter.

Chapter II

Purification and characterization of molybdenum-containing aldehyde dehydrogenase that oxidizes benzyl maltol derivative from *Pseudomonas nitroreducens* SB32154

In chapter I, the author reported that cytochrome P450 monooxygenase, P450_{nov}, which had the ability to effectively convert the 2-methyl group in a maltol derivative, transformed 2-methyl-3-(phenylmethoxy)-4*H*-pyran-4-one (BMAL) to 2-(hydroxymethyl)-3-(phenylmethoxy)-4*H*-pyran-4-one (BMAL-OH) and slightly to 3-benzoyloxy-4-oxo-4*H*-pyran-2-carboxaldehyde (BMAL-CHO) .

In chapter II, the author focused on oxidation of BMAL-OH and examined the development of biocatalysts to oxidize a 2-hydroxymethyl side chain of BMAL-OH. The 2-carboxylic acid group in the final reaction product could be a chemical modifying point for expanding the functional diversity of maltol derivatives. BMAL-CHO dehydrogenase was identified as the enzyme responsible for BMAL-CHO oxidation in *Pseudomonas nitroreducens* SB32154, and characterized the purified enzyme (Fig. 2-1).

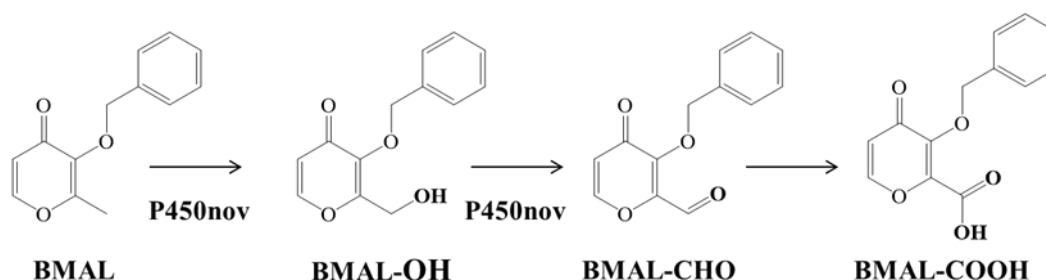


Fig. 2-1 Diagram of BMAL-COOH production from BMAL

Materials and methods

Materials

BMAL-OH, BMAL-CHO, and BMAL-COOH were synthesized as previously described (25, 36). Soil samples were collected in Kinki region of Japan.

Enrichment for BMAL-OH-assimilating microorganisms

Approximately 1 mg of each soil sample was suspended in 1 ml of M9 minimal medium in 96-well assay blocks (1 ml; Corning, NY, USA). The suspensions were sonicated and centrifuged, and then the supernatants were added to 0.5 ml of enrichment medium (pH7.0) containing 11.28 g/L M9

minimal salts (5 × powder), 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.7 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 ml/L metal mixture (0.1 g/L H_3BO_3 , 0.5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L KI, 2 g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 4 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/L H_2SO_4), and 500 mg/L BMAL-OH as a sole carbon source, in 96-well assay blocks (1 ml; Corning). Enrichment cultivation was carried out in a 96-well plate shaker at 30 °C, and 10% of the volume of the culture was transferred to fresh enrichment medium every 2 days. After passaging twice, the resultant culture was centrifuged, and the supernatant was mixed with an equal volume of ethanol. BMAL-OH metabolites in the solution were analyzed by HPLC. The BMAL-COOH-producing microorganisms were stored in Nutrient Agar Broth (Becton Dickinson, NJ, USA).

Screening of BMAL-OH and BMAL-CHO-oxidizing microorganisms

The BMAL-OH-assimilating microorganisms were inoculated into BMAL-OH or BMAL-CHO medium containing 11.28 g/L M9 minimal salts (5 × powder), 2 g/L succinate, 0.25 g/L yeast extract, 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 14.7 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 ml/L of metal mixture as described above, and 200 mg/L BMAL-OH or BMAL-CHO, and were cultivated in a 96-well plate shaker at 30 °C. After cultivation for 7 days, the culture was centrifuged, and the supernatant was mixed with an equal volume of ethanol. BMAL-COOH in the solution was analyzed by HPLC. Single colony isolation of BMAL-COOH-producing strains was carried out on Nutrient Agar Broth. Microbial identification was carried out by 16S rDNA sequence analysis using a MicroSeq Full Gene 16S rDNA Bacterial Identification kit (Applied Biosystems, CA, USA).

Assay of BMAL-CHO dehydrogenase

BMAL-CHO dehydrogenase activity was assayed by the method of Mohapatra et al. (37) with some modifications. The standard assay mixture containing 15 µl of 20 mM phosphate buffer (pH 6.0), 0.25 µl of 20 mg/ml BMAL-CHO in DMSO, 0.3 µl of 20 mM 2,6-dichlorophenolindophenol (DCIP) and 0.5 µl of 20 mM phenazine methosulfate (PMS). The reaction was started by the addition of 5 µl of enzyme solution in a total volume of 21 µl and carried out at 30 °C for 30min. The rate of decrease in the absorbance at 600 nm was followed against a blank in which gel filtration buffer was added in place of enzyme. One unit (U) of BMAL-CHO dehydrogenase activity was defined as the 1 µmol of DCIP reduced per minute per liter of enzyme solution. Protein concentration was quantified by using *Pierce Coomassie Protein Assay kit* (Thermo Fisher Scientific, MA, USA) with bovine serum albumin standard.

Purification of a BMAL-CHO-oxidizing enzyme from *Pseudomonas nitroreducens* SB32154

For purification of BMAL-CHO-oxidizing enzyme, *Pseudomonas nitroreducens* SB32154 was cultivated in 1 L LB medium containing 10 ml ethanol. Cultivation was carried out at 30 °C for 24h with agitation of 80 rpm on a rotary shaker. The bacterial cells were harvested by centrifugation at 5,500 × g

for 15min at 4 °C. The resulting cell pellets were resuspended in 20 mM Tris-HCl buffer (pH8.0) containing 2 mM DTT and disrupted with a probe sonicator (Branson Sonifier 450; Emerson, MO, USA). The parameter was set at output of 6 at 20% duty cycle in the 10 s pulse mode for 30 min. The cell debris was removed by centrifugation at 8,000 x *g* for 30 min at 4 °C. The 0.22 µm-filtrated cell-free supernatant was designated as crude BMAL-CHO dehydrogenase. All purification procedures were performed at 4 °C using an AKTA FPLC system (GE Healthcare, Little Chalfont, UK).

The crude BMAL-CHO dehydrogenase was applied to a HiPrep DEAE 16/10 FF column (GE healthcare) previously equilibrated with 20 mM Tris-HCl buffer (pH8.0), which was used as a standard buffer for following purification steps. After the column had been washed with 40 ml of this buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.4 M) in 100 ml of 20 mM buffer. The active fractions were combined, dialyzed against 5 L of 20 mM buffer for 24 h using Slide-A-Lyze 10 kDa molecular weight cutoff cassettes (*Thermo Fisher Scientific*). The enzyme solution was applied to MonoQ 5/50 GL column (GE healthcare) previously equilibrated with 20 mM buffer. After the column had been washed with 2 ml of 20 mM buffer, the enzyme was eluted with a linear gradient of NaCl (0–0.3 M) in 20 ml of 20 mM buffer. The active fractions were combined, added same volume of 1.6 M (NH₄)₂SO₄, and then applied to HiTrap Butyl HP 5 ml column (GE healthcare) previously equilibrated with 20 mM buffer containing 0.8M (NH₄)₂SO₄. The enzyme was eluted with a linear gradient of (NH₄)₂SO₄ (0.8–0 M) in 100 ml of 20 mM buffer. The active fractions were combined, concentrated, and exchanged the buffer to 20 mM buffer with 50 mM NaCl by ultrafiltration (Amicon Ultra; Merck Millipore, MA, USA). The enzyme solution was applied to a Superdex 200 HR 10/30 column (GE healthcare) previously equilibrated with 20 mM buffer containing 50 mM NaCl and eluted with the same buffer. The active fractions were used for characterization. Amino acid sequence analysis of the amino terminus of the purified enzyme was performed with a Procise 494 HT protein sequencing system (*Thermo Fisher Scientific*).

Characterization of BMAL-CHO dehydrogenase

The effect of different electron acceptors was evaluated with the purified enzyme from *P. nitroreducens* SB32154 in the standard reaction mixture with each electron acceptor instead of DCIP and PMS. The reaction was carried out at 30 °C for 30 min. The enzyme activity was evaluated by the production of BMAL-COOH using LC-MS analysis.

To determine the substrate specificity, the assay conditions were the same as mentioned above except different aldehydes were used as the substrate instead of BMAL-CHO. Separate blanks with individual substrates of aldehyde were also prepared. The kinetics parameters, such as Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) values against aldehyde substrates were calculated using XLfit (IDBS, Guilford, UK)

Cloning and expression of recombinant BMAL-CHO dehydrogenase in *E. coli*

Primers were designed to amplify the BMAL-CHO dehydrogenase sequence, without a stop codon, from *P. nitroreducens* SB32154 genomic DNA. The PCR-amplified product was ligated into expression vector pET11a (Novagen, WI, USA), using the restriction enzyme site of *Nde*I and *Xho*I. The resulting plasmid was purified, and then used to transform *E. coli* BL21 STAR (DE3) (Thermo Fisher Scientific, MA, USA). The transformed cells were cultured in Luria-Bertani (LB) medium at 37 °C for 2 h with shaking at 150 rpm, and then isopropyl- β -thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After adding IPTG, the transformed cells were cultivated at 25 °C for 24 h with shaking at 150 rpm. The profile of protein expression was checked on denaturing SDS-PAGE. The activity of BMAL-CHO conversion was checked with disrupted recombinant *E. coli* cells by method indicated above and BMAL-COOH production was measured by LC-MS.

One-pot reaction from BMAL to BMAL-COOH

P450nov and redox partner proteins were expressed in *E. coli* by the method of the author's previous paper (39). For preparing P450nov expressed cell suspension, 1 ml of expressed recombinant *E. coli* was centrifuged and suspended in 500 μ l of phosphate-buffered saline (PBS). Ninety microlitter of recombinant *E. coli* suspension and 10 μ l of purified BMAL-CHO dehydrogenase from *P. nitroreducens* SB32154 were mixed, and added 0.5 μ l of 50 mg/ml BMAL in DMSO. The reaction was carried out at 28 °C for 20 h with agitation of 1,400 rpm and BMAL-COOH production was measured by LC-MS analysis.

Results

Screening of microorganisms producing BMAL-COOH from BMAL-OH

From a total of 665 soil samples, BMAL-COOH-producing microorganisms were screened with the enrichment medium using BMAL-OH as a sole carbon source. Out of these, 3 samples converted BMAL-OH to BMAL-COOH. From these samples, BMAL-OH-oxidizing strains were not isolated, while 3 BMAL-CHO-oxidizing strains were isolated. According to the 16S rDNA sequence analysis, these BMAL-CHO-oxidizing strains were identified as *Pseudomonas nitroreducens*. The strain with the highest activity, *Pseudomonas nitroreducens* SB32154 (NBRC114008), produced 85 mg/L BMAL-COOH from 100 mg/L BMAL-CHO after 24 h, and was chosen for further investigation.

Identification of a BMAL-CHO-oxidizing enzyme of *P. nitroreducens* SB32154

The course of purification is summarized in Table 1. After 4-step purification procedure, the enzyme purity was increased approximately 139-fold (Table 2-1). The purified enzyme showed two major bands on SDS-PAGE (Fig. 2-2). The molecular masses of these proteins were about 75,000 and 15,000. Amino acid sequence analysis of these proteins revealed the N-terminal amino acid sequence of

upper protein band of DFEFNAFVXRIAPDGTVT and that of lower protein band of MITVNLNGKDHELDAPGEM. These sequences matched that of a putative xanthine dehydrogenase family protein molybdopterin-binding subunit from *Pseudomonas nitroreducens* and that of a putative (2F-2S)-binding protein from *Pseudomonas* sp., respectively. To indentify the nucleotide sequences of these proteins, we determined the genome sequence of *P. nitroreducens* SB32154, and identified the full-length amino acid sequences of the two subunits of BMAL-CHO-oxidizing enzyme (GenBank accession number: LC480925, LC480926). Those sequences showed 99% identical to those of corresponding putative xanthine dehydrogenase from *P. nitroreducens* (WP_088417191, WP_026078846).

Table 2-1 Purification table of BMAL-CHO dehydrogenase from *P. nitroreducens* SB32154

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Cell-free extract	562	5.10	0.01	1
Hiprep DEAE 16/10 FF	17.5	0.19	0.01	1
MonoQ 5/50 GL	1.50	0.15	0.10	11
Hitrap butyl FF	0.19	0.10	0.50	55
Superdex 200 10/300 GL	0.04	0.05	1.26	139

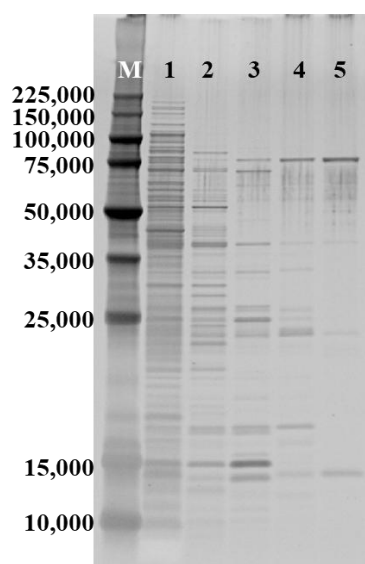


Fig. 2-2 SDS-PAGE of enzyme fractions of each purification step

Lane M: broad range protein molecular weight markers (Promega, WI, USA), Lane 1: cell-free extract, Lane 2: fraction after weak ion exchange chromatography, Lane 3: fraction after strong ion exchange chromatography, Lane 4: fraction after hydrophobic interaction chromatography, Lane 5: purified enzyme after gel-filtration chromatography

Characterization of a BMAL-CHO-oxidizing enzyme of *P. nitroreducens* SB32154

PMS served as an electron acceptor effectively (Fig. 2-3). Methylene blue, DCIP, and ferricyanide also served as an electron acceptor but less effectively than PMS.

Various aldehydes were evaluated as the substrates of purified BMAL-CHO dehydrogenase of *P. nitroreducens* SB32154 by measuring the reduction of DCIP (Table 2-2). The enzyme showed broad substrate specificity to benzaldehyde analogues, but did not accept purines and pyrimidines as a substrate.

Kinetics parameters, i.e., the Michaelis-Menten constant (K_m) and maximum reaction velocity were determined for purified BMAL-CHO dehydrogenase with some aldehyde and PMS. The kinetic analysis revealed that this aldehyde dehydrogenase has an apparent K_m of 25 mM for BMAL-CHO (Table 2-3).

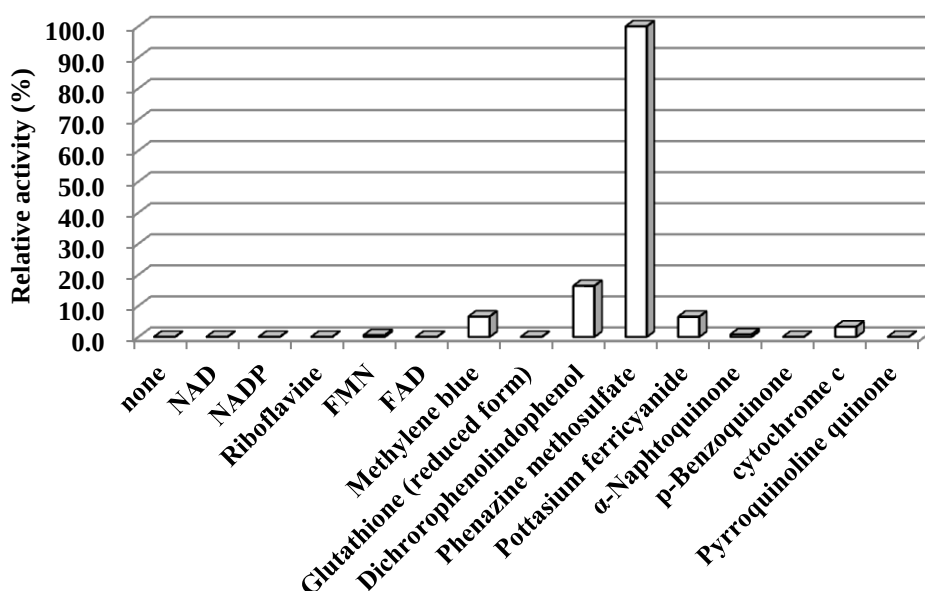


Fig. 2-3 Evaluation of BMAL-CHO dehydrogenase activity with various electron acceptors

Relative activities of BMAL-CHO dehydrogenase using various electron acceptors were presented, which were calculated based on BMAL-COOH production measured by peak area of 247 m/z mass spectrum, relative to using PMS as an electron acceptor. The reactions were carried out with 20 μ l of the reaction mixture containing 37.8 ng of the purified enzyme, 0.25 μ l of 20 mg/ml BMAL-CHO in DMSO, and 0.5 μ l of 20 mM electron transporter, at pH 6.0, 30 $^{\circ}$ C, for 30 min.

Table 2-2 Substrate specificity of BMAL-CHO dehydrogenase

Substrate	Relative activity (%)
BMAL-CHO	100
benzaldehyde	311
<i>o</i> -hydroxybenzaldehyde	159
<i>m</i> -hydroxybenzaldehyde	103
<i>p</i> -hydroxybenzaldehyde	81.1
<i>o</i> -nitorobenzaldehyde	132
<i>m</i> -nitorobenzaldehyde	296
<i>p</i> -nitorobenzaldehyde	364
<i>o</i> -phthalaldehyde	80.2
Terephthalaldehyde (<i>p</i> -phthalaldehyde)	319
vanillin	132
cinnamaldehyde	53.2
isoquinoline	n.d.
adenine	n.d.
hypoxanthine	n.d.
xanthine	n.d.
guanine	n.d.
thymine	n.d.
cytidine	n.d.
uracil	n.d.
acetoaldehyde	n.d.
formaldehyde	n.d.

Relative activities of various aldehyde substrates were presented, which were calculated based on DCIP reduction relative to BMAL-CHO. The reactions were carried out with 20 µl of the reaction mixture containing 200 ng of the purified enzyme, 0.25 µl of 100 mM substrates in DMSO, 0.5 µl of 20 mM PMS, and 0.3 µl of 20 mM DCIP, at pH 6.0, 30 °C, for 30 min. n.d., not detected.

Table 2-3 K_m and V_{max} values of BMAL-CHO dehydrogenase from *P. nitroreducens* SB32154

Substrate	V_{max} (U/mg)	K_m (mM)	V_{max}/K_m (U/mg/mM)
BMAL-CHO	0.36	24.0	0.01
benzaldehyde	0.46	6.95	0.07
<i>o</i> -hydroxybenzaldehyde	0.30	1.02	0.30
<i>m</i> -hydroxybenzaldehyde	0.30	1.08	0.28
<i>p</i> -hydroxybenzaldehyde	0.11	0.75	0.14
<i>o</i> -nitorobenzaldehyde	0.17	0.17	0.99
<i>m</i> -nitorobenzaldehyde	0.35	0.22	1.57
<i>p</i> -nitorobenzaldehyde	0.37	0.35	1.06
cinnamaldehyde	0.20	1.50	0.13

K_m and V_{max} values of various aldehydes, which were calculated based on DCIP reduction, were presented. The reactions were carried out with 20 μ l of the reaction mixture containing 62 ng of the purified enzyme, 0.25 μ l of substrates in DMSO, 0.5 μ l of 20 mM PMS, and 0.3 μ l of 20 mM DCIP, at pH 6.0, 30 °C, for 30 min.

Cloning and expression of recombinant BMAL-CHO dehydrogenase in *E. coli*

BMAL-CHO dehydrogenase was consisted of two subunits, i.e., molybdopterin subunit and (2Fe-2S)-binding subunits. The enzyme was expressed in *E. coli* by transformation with a vector having these two subunit genes tandemly after T7 promoter in order of the genome sequence. Each protein was expressed (Fig. 2-4) and BMAL-COOH production from BMAL-CHO by the transformant was confirmed in comparison with that of the host *E. coli* cell (Fig. 2-5).

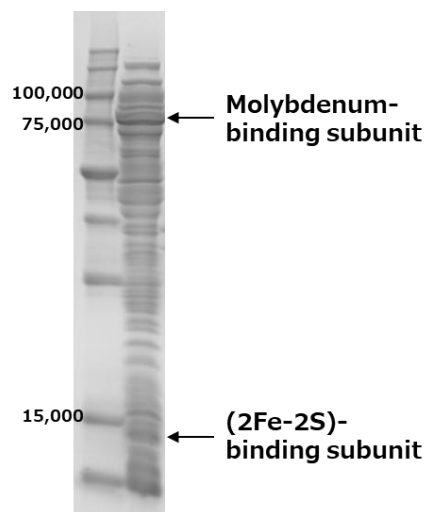


Fig. 2-4 SDS-PAGE analysis of recombinant BMAL-CHO dehydrogenase in *E. coli*

Molecular weight of expressed molybdenum-binding subunit was 75 kDa and that of (2Fe-2S)-binding subunit was 15 kDa.

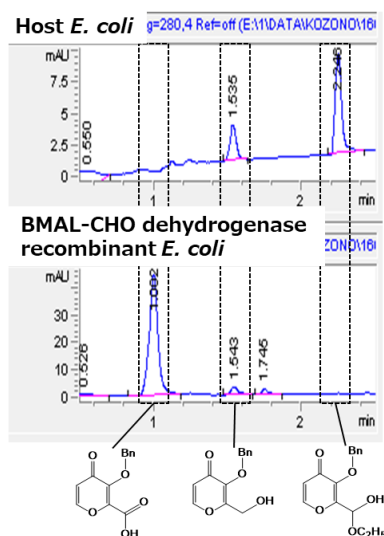


Fig. 2-5 BMAL-CHO conversion of BMAL-CHO dehydrogenase recombinant *E. coli*

The reactions were carried out with 20 μ l of the reaction mixture containing 10 μ l of the disrupted BMAL-CHO dehydrogenase-expressing recombinant *E. coli* solution, 0.25 μ l of 20 mg/ml BMAL-CHO in DMSO, 0.5 μ l of 20 mM PMS, and 0.3 μ l of 20 mM DCIP, at pH 6.0, 30 $^{\circ}$ C, for 30 min. BMAL derivative production was measured by 280 nm absorbance. Upper chart indicates the reduction of BMAL-CHO by host *E. coli* cell. Lower chart indicates the production of BMAL-COOH by BMAL-CHO dehydrogenase-expressing recombinant *E. coli*. The peak at 2.2 min in the chart indicates ethanol adducted hemiacetal form of BMAL-CHO.

One-pot production of BMAL-COOH from BMAL by using P450nov and BMAL-CHO dehydrogenase

For one-pot enzymatic production of BMAL-COOH from BMAL, combination with P450nov and BMAL-CHO dehydrogenase was evaluated. When purified BMAL-CHO dehydrogenase of *P. nitroreducens* SB32154 was combined with the cell suspension of *E. coli* expressing P450nov (L188P/F218L), FDXnov, and FDRnov, a significant amount of BMAL-COOH was produced from BMAL as the substrate (Fig. 2-6).

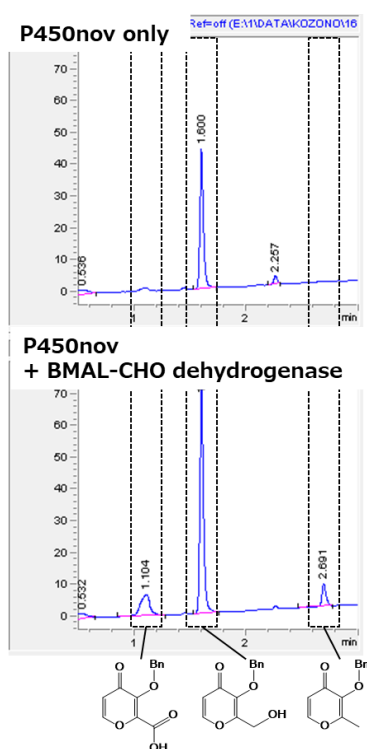


Fig. 2-6 One-pot enzymatic conversion of BMAL to BMAL-COOH

The reactions were carried out with 90 μ l of P450nov-expressing recombinant *E. coli* suspension, 150 ng of purified BMAL-CHO dehydrogenase of *P. nitroreducens* SB32154, and 0.5 μ l of 50 mg/ml BMAL, at 28 $^{\circ}$ C for 20 h. BMAL derivative production was measured by 280 nm absorbance. Upper chart indicates the conversion of BMAL using only P450nov. Lower chart indicates conversion of BMAL using P450nov and BMAL-CHO dehydrogenase.

Discussion

In chapter II, the author reported the aldehyde dehydrogenase from *Pseudomonas nitroreducens* SB32154 which catalyzes oxidation of BMAL-CHO to BMAL-COOH. This enzyme was a member of xanthine dehydrogenase family protein and consisted of two different subunits, i.e., iron-sulfur-binding subunit and molybdenum-binding protein subunit. Xanthine oxidoreductase family protein exists in a great variety of organisms from bacteria to higher plants and human, and generally consisted of three different subunits, N-terminal iron-sulfur-binding subunit, intermediate FAD-binding subunit, and C-terminal molybdenum-binding subunit. Electrons that are passed to the molybdenum during the oxidation are transferred to FAD via the iron sulfur centers. Finally, NAD^+ or oxygen molecule, which is the final electron acceptor, is reduced (38). On the other hand, this BMAL-CHO dehydrogenase, which did not contain FAD, could not use NAD^+ or molecular oxygen as the final electron acceptor. Addition of flavin derivative, riboflavin, FMN, and FAD, to the purified enzyme did not increase BMAL-CHO oxidation activity, but methylene blue, DCIP, potassium ferricyanide, cytochrome c, and especially PMS, succeeded in accepting electrons and increased BMAL-COOH production. The native final electron acceptor has not been revealed, but the bacterial respiratory chain may have been involved because of its ability of using cytochrome c as electron acceptor. Xanthine dehydrogenase family protein lacking FAD-binding subunit have also been reported from *Pseudomonas diminuta* 7 (40) and *Desulfovibrio gigas* (41). These enzymes exhibited similar substrate specificity such as benzaldehyde was good substrate but xanthine was not. Isoquinoline 1-oxidoreductase from *P. diminuta* 7 could hydroxylate N-heterocyclic compound, but BMAL-CHO dehydrogenase could not. Aldehyde oxidoreductase from *D. gigas* could not use PMS as an electron acceptor, but BMAL-CHO dehydrogenase could. From such difference, BMAL-CHO dehydrogenase in this study could be a novel xanthine dehydrogenase family protein which did not have intermediate FAD-binding subunit.

In this chapter, the author also achieved one-pot enzymatic conversion of BMAL to BMAL-COOH by combining the BMAL-CHO dehydrogenase with P450nov, which could catalyze BMAL to BMAL-OH and had weak activity of BMAL-OH oxidation to BMAL-CHO. But the conversion efficiency of this one-pot reaction was low, because BMAL-OH was accumulated during the reaction and only a small amount of BMAL-COOH was produced from BMAL as substrate (Fig. 2-6). BMAL-OH accumulation was caused by the BMAL-CHO reduction activity of host *E. coli* cell (Fig. 2-5) and weak BMAL-OH oxidation activity of P450nov. To reduce BMAL-CHO reduction activity, it is necessary to knock out the corresponding enzyme in host *E. coli* cell. And to improve the BMAL-OH oxidation activity, P450nov are needed to be evolved or novel alcohol oxidoreductase which can convert BMAL-OH to BMAL-CHO are needed to be found. As for BMAL-CHO dehydrogenase, the affinity for BMAL-CHO was very low ($K_m=25\text{ mM}$) compared to benzaldehyde derivatives (Table 2-3). It may be

possible to improve substrate specificity if appropriate mutations were introduced into BMAL-CHO dehydrogenase. As mentioned above, by improving the components of this one-pot enzymatic conversion BMAL to BMAL-COOH via BMAL-CHO, the author expect to produce BMAL-COOH more effectively, which have potential applications in various fields.

Summary

In chapter II, microbial enzymes useful for BMAL-OH oxidation to BMAL-COOH via BMAL-CHO were screened. *Pseudomonas nitroreducens* SB32154 was found to have the ability to convert BMAL-CHO to BMAL-COOH. The enzyme responsible for the aldehyde oxidation, a BMAL-CHO dehydrogenase, was purified from *P. nitroreducens* SB32154 and characterized. The purified BMAL-CHO dehydrogenase was found to be a xanthine dehydrogenase (XDH) family enzyme with unique structure of hetero dimer which was composed of 75 kDa and 15 kDa subunits containing a molybdenum-cofactor and [Fe-S] clusters, respectively. The enzyme showed broad substrate specificity towards benzaldehyde derivatives but was not able to oxidize purine bases, xanthine, and hypoxanthine. The K_m and V_{max} for BMAL-CHO were 24.97 mM and 0.36 U/mg of protein, respectively. Furthermore, one-pot conversion of BMAL to BMAL-COOH via BMAL-CHO by combined action of the BMAL-CHO dehydrogenase with P450nov was achieved.

Chapter III

Characterization of xanthine oxidase from *Cellulosimicrobium funkei* possessing purine base-metabolizing activity

In chapter III, the author proposed a new prophylaxis for hyperuricemia applying enzymes derived from microorganisms (Fig. 3-1). The author described about the screening of microorganisms which decomposed hypoxanthine which is known to have potency of increasing serum uric acid level in purine bases. Through the screening, hypoxanthine degrading microorganisms were isolated, and the enzyme involved in the metabolism was purified, cloned, and characterized. The enzyme could oxidize hypoxanthine, an easily adsorbed purine, to xanthine and further to uric acid, hardly adsorbed purines, and promising as a tool for hyperuricemia medication.

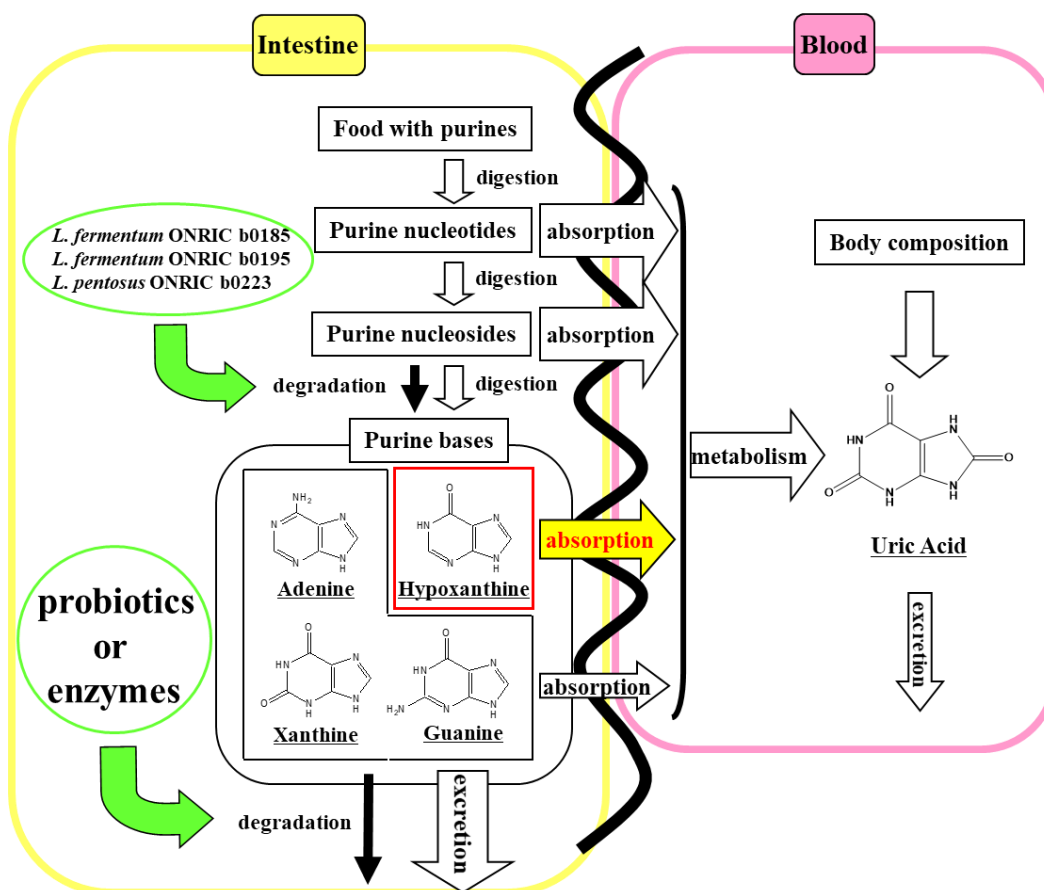


Fig. 3-1 Proposed mechanism of lowering uric acid level in blood by probiotic bacteria and enzymes

Materials and methods

Materials

Hypoxanthine was purchased from WAKO Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals used in this work were of analytical grade and commercially available.

Isolation of hypoxanthine-assimilating microorganisms

Hypoxanthine-assimilating microorganisms were isolated from soils collected in Kyoto city, Japan. A little amount of soil samples were added into a test tube (20 × 130 mm) containing 5 ml of isolation medium comprising 0.1 % (w/v) KH_2PO_4 , 0.1 % (w/v) K_2HPO_4 , 0.03% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 % (w/v) yeast extract, 0.2% (w/v) NH_4Cl , and 0.15% (w/v) hypoxanthine (pH 7.0). The culture medium was incubated aerobically with shaking (300 strokes/min) at 37 °C for 3–5 days. The medium (50 μl), in which growth of microorganisms were observed, was transferred into another test tube containing the isolation medium and further cultivated under the each condition for 3–5 days. These culture media were streaked onto agar plates with the same composition as isolation medium and cultivated at 37 °C for 3–5 days. After the cultivation, the colonies appeared were isolated as hypoxanthine-assimilating microorganisms and these strains were used for following experiments.

First screening

Aerobic hypoxanthine-assimilating microorganisms were inoculated into 5 ml of the isolation medium and cultivated at 37 °C for 3 days. After the incubation, the culture broths were diluted 3 fold by adding dimethyl sulfoxide (DMSO). The cells were collected by centrifugation at 10,000 rpm for 10 min (LX-31, Tomy Seiko, Tokyo, Japan). Then the supernatants were analyzed by HPLC as described below.

Second screening

Strains isolated from soils with confirmed hypoxanthine-degrading activity in the 1st screening were used. Reaction mixture (200 μl) comprising 25 mM hypoxanthine, 200 mM buffer (Sodium acetate-HCl; pH 4.5, Tris-HCl; pH 9.5, or none; pH 8.0), 0.4% (v/v) polyoxyethylenelaurylamine, 1% (v/v) xylene, 5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 50 % (wet weight/v) washed cells was added into the eppendorf tube and incubated at 37 °C with shaking (300 strokes/min) for 2 days. After reaction, the reaction mixtures were diluted 3 times by adding DMSO, and then centrifuged at 10,000 rpm for 10 min (LC-100, Tomy Seiko, Tokyo, Japan). The supernatants were analyzed by HPLC.

Third screening

Hypoxanthine-degrading activity of the selected strains in the 2nd screening were evaluated using cell-free extracts (CFEs) disrupted by sonication. Reaction mixture (200 μl) comprising 0.5 mM hypoxanthine (in 25 mM NaOH), 200 mM Tris-HCl buffer (pH 7.4), and 1.25 % (v/v) CFEs were added into the eppendorf tube and incubated at 37 °C with shaking (150 shakes/min) for 10 min. After the

reaction, the reaction mixtures were centrifuged at 10,000 rpm for 10 min and the supernatants were analyzed by HPLC.

HPLC analysis

Quantitative analysis of hypoxanthine-degradation was performed by reverse-phase high-performance liquid chromatography (HPLC) by using a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C18 PAQ column (250 × 4.6 mm, Nacalai tesque Co., Ltd, Japan). The mobile phase was 50 mM K₂HPO₄ (pH 2.0) at a flow rate of 1.0 ml/min and the effluents were monitored by ultraviolet (UV) detection at 254 nm. The injection volume was 10 µl.

Identification of selected strains

After the three stages of screening, one strain isolated from soils was selected and identified by TechnoSuruga Laboratory Co. Ltd based on 16S rDNA analysis.

Analysis of purine metabolism

Purine-decomposing activity was evaluated by using resting cells. Reaction mixture (300 µl) comprising 1 mM purine (in 25 mM NaOH), 200 mM buffer (Tris-HCl; pH 7.4), 0.1 % (v/v) polyoxyethylenelaurylamine, 0.3 % (v/v) xylene, 1.7 mM MgSO₄·7H₂O and 1 % (wet weight/v) washed cells was added to the eppendorf tube and incubated at 37 °C with shaking (150 strokes/min) for 24 h. After the reaction, the reaction mixtures centrifuged at 10,000 rpm for 10 min. The supernatants were analyzed by HPLC.

Enzyme assay

Enzyme activity was determined by the decreased amount of substrate (hypoxanthine; 255 nm) and increasing amounts of products (xanthine, 275 nm; uric acid, 290 nm) measured by spectrophotometric analysis using micro plate reader (Molecular Device, Concord, Canada). Reaction mixture (180 µl) comprising 0.2 mM hypoxanthine (in 25 mM NaOH) and 200 mM Tris-HCl (pH 7.4) was added to the each well of 96-well plate and the reaction was started by adding the appropriate amounts of the enzyme. Then changes in absorbance were monitored at 3 wave lengths (255 nm, 275 nm, and 290 nm) at 37 °C for 30 min.

Enzyme Purification

All steps were carried out at 0 to 5 °C and the buffer used was 20 mM Tris-HCl (pH 7.4), unless stated otherwise. Fractions were analyzed with their activity by spectrophotometric analysis as described above and all active fractions were subjected to SDS-PAGE analysis as described below.

Step 1

Cellulosimicrobium funkei A103 was cultivated in 10 L of the isolation medium supplemented with 0.2 % (w/v) meat extract at 37 °C for 2 days and harvested by centrifugation (10,000 rpm at 4 °C). The resulting wet cells (43.8 g) were suspended in 75 ml of buffer and disrupted with glass beads 0.25 to 0.50 mm in diameter (Dyno-Mill KDI., W. A. Bachofen, Switzerland) at 5 °C for 30 min. The disrupted cell suspension was filtered and then centrifuged at 10,000 rpm for 30 min at 4 °C. The resultant supernatant was used as cell-free extracts.

Step 2

The cell-free extracts were dialyzed against 8 L of buffer for 12 h. The dialyzed sample was then applied to ammonium sulfate fractionation (0–40, 40–60, 60–80 %). The reaction of 40–60 % was dialyzed and filtered using MILLEX GV (MILLIPORE, Massachusetts, USA).

Step 3

The filtered enzyme was applied to a Hiprep 16/10 DEAE FF column equilibrated with buffer and then eluted with a linear gradient of 0 to 1 M NaCl in 200 ml of buffer. The active fractions were combined and dialyzed.

Step 4

The dialyzed enzyme was applied to a Mono Q HR 10/10 column equilibrated with buffer and then eluted with a linear gradient of 0 to 1 M NaCl in 50 ml of buffer. The active fractions were combined and dialyzed.

Step 5

Solid (NH₄)₂SO₄ was added to the dialyzed enzyme solution in order to obtain a concentration of 1.5 M. The resulting enzyme solution was applied to a Phenyl Superose HR 5/5 column equilibrated with the buffer containing 1.5 M (NH₄)₂SO₄. The enzyme was eluted with decreasing (NH₄)₂SO₄ concentration from 1.5 to 0 M in 20 ml of buffer. The active fractions were combined and dialyzed.

Step 6

The dialyzed enzyme was applied to a Mono Q HR 5/5 column equilibrated with buffer and then eluted with gradients from 0 to 0.6 M NaCl in 20 ml of buffer. The active fractions were combined and concentrated by ultrafiltration (Amicon Ultra-4, MILLIPORE, Massachusetts, USA).

Step 7

The concentrated enzyme was applied to a Superdex 200 PC 3.2/30 column equilibrated with buffer containing 0.2 M NaCl and then eluted in 3 ml of buffer. The active fractions were combined and concentrated by ultrafiltration.

Step 8

The concentrated enzyme was applied to a Mono Q PC 1.6/5 column equilibrated with buffer and then eluted with gradient from 0.3 to 0.4 M NaCl in 3.2 ml of buffer. The active fractions were combined.

Protein analysis

The protein concentration was measured with a protein assay kit (BIORAD, California, USA) using BSA as standard. SDS-PAGE was performed on a 12.5 % polyacrylamide slab gel with Tris-glycine buffer system. The molecular mass of the subunit was determined from its mobility relative to those of the standard proteins. Protein bands were stained with Coomassie Brilliant Blue R250.

NH₂-terminal and internal amino acid sequences of purified enzyme were determined by automated Edman degradation with a peptide sequencer 476 (Applied Biosystem, California, USA).

***De novo* assembly of the *Cellulosimicrobium funkei* A153 genome**

Extracted genomic DNA of *C. funkei* A153 was subjected to library construction using the Nextera DNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA). Library quality was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The library was sequenced on Illumina MiSeq (300-bp paired-end sequence). The paired-end reads were quality-checked with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and trimmed using Trimmomatic (LEADING:32, TRAILING:32, MINLEN:100, SLIDINGWINDOW:4:

30) (Reference PMID: 24695404). The trimmed reads were assembled using the A5-miseq pipeline (Reference PMID: 25338718), and the assembled genome was annotated by the RAST server (Reference PMID: 18261238).

Construction of strains for expression of xanthine oxidase

The XOD gene (Accession number: LC504731) was amplified by PCR using the following primers: 5'-TGTTTAACTTTAAGAAGGAGATATACCATGGACCTGGGTACCGTCACC-3' and 5'-TGGTGATGGTGATGCTCGAGAGATCTGCGGGTGGGGGTGGCGGC-3', which were designed for expression of his-tagged xanthine oxidase. PCR was carried out with Tks Gflex DNA polymerase (Takara, Shiga, Japan) under the following conditions: 94 °C for 1 min and 30 cycles of 98 °C for 10 sec, 60 °C for 15 sec, and 68 °C for 4 min. An expression vector for *Rhodococcus*, pTipQC1 (Hokkaido System Science, Hokkaido, Japan), was digested with HindIII and NcoI, purified by gel electrophoresis, mixed with the PCR products, and ligated using NEBuilder HiFi DNA Assembly (New England Biolabs, Massachusetts, USA). The resultant plasmid, pTipQC1/His-tag-XOD, was transformed into *Rhodococcus erythropolis* L88 (Hokkaido System Science, Hokkaido, Japan).

Expression and purification of recombinant XDH

The *Rhodococcus erythropolis* pTipQC1/His-tag-XOD was inoculated in 5 mL of pre-culture LB medium with 25 µg/mL of chloramphenicol and cultivated at 28 °C with shaking at 300 rpm for overnight. The resulting pre-culture medium was inoculated into 90 mL of LB medium with 25 µg/mL of chloramphenicol and cultivated at 28 °C with shaking at 120 rpm for 3 h. After 3 h, 0.2 µg/mL of

thiostrepton was added to the culture medium and cultivated again at 28 °C for 1 day. The transformed cells in total 200 mL of culture broth were harvested, suspended in the binding buffer described below (10 mL) with 15 mg of lysozyme and treated with an ultrasonic oscillator (30 min, Insonator 201 M; Kubota, Japan). The binding buffer contains 20 mM imidazole, 0.5 M NaCl, 1 mM dithiothreitol (DTT), and 20 mM Tris-HCl (pH 7.4). The cell debris was removed by centrifugation at 1,700 g for 15 min. The resulting supernatant solutions were used as cell-free extracts. The cell-free extracts were filtered with 0.45 µm Millex-HPF HV membrane filter (Merck Millipore, Massachusetts, USA). The enzyme was purified from the cell-free extracts by the fast protein liquid chromatography (FPLC) system (Amercham Pharmacia Biotech Co., Uppsala, Sweden) with His Trap HP (GE Healthcare, Buckinghamshire, England). FPLC was equilibrated with binding buffer and the fractions containing XOD were eluted by elution buffer. The elution buffer contains 0.5 M imidazole, 0.5 M NaCl, 1 mM DTT, and 20 mM Tris-HCl (pH 7.4). The fractions containing XOD were collected, desalted, and concentrated with Vivaspın Turbo 15 (Sartorius, Gottingen, Germany). The buffer was replaced with 20 mM Tris-HCl (pH 7.4) containing 1 mM DTT. All procedures were carried out at 4 °C.

Electron acceptor specificity

The activity of the purified enzyme was evaluated by generation of uric acid in the presence of various kinds of electron acceptors. Reaction mixture (200 µl) comprising 0.1 mM xanthine, 200 mM potassium phosphate buffer (KPB) (pH 7.0), 0.5 mM each electron acceptor, and appropriate amounts of purified enzyme was incubated at 45 °C. The initial velocity to generate uric acid was detected at 290 nm with UV spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan).

Substrate specificity

H₂O₂ was produced by two-electron reduction of O₂ accompanied with the oxidation of substrates by the xanthine oxidase. Substrate specificity of the purified enzyme was evaluated by color development resulted from peroxidase catalyzing oxidative condensation of 4-aminoantipyrine (4-AA) and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS) in the presence of H₂O₂ generated by the xanthine oxidase reaction. Reaction mixture (185 µl) comprising 0.2 mM substrates, 162 mM Tris-HCl (pH 7.4), 0.6 mM 4-AA, 0.7 mM TOOS, 2000 U peroxidase from horseradish (Oriental Yeast Co., Ltd., Tokyo, Japan), and appropriate amounts of the purified enzyme was mixed and change of absorbance at 550 nm was monitored at 45 °C with UV spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan). The products were identified by LC-MS as described below.

Identification of the products

The products were analyzed by electrospray ionization mass spectrometry (ESI-MS) using a LCMS-2020 (Shimadzu, Kyoto, Japan). The conditions were as follows: block temperature, 200 °C;

desolvation line temperature, 250 °C; detector voltage, 1.5 kV; nebulizing gas flow, 1.5 L/min.

Effects of reaction conditions and enzyme stability

To evaluate the optimum temperature and pH for the purified xanthine oxidase activity, the enzyme assay was performed at different temperatures from 20 to 70 °C with 10°C increments and with buffers of different pH, such as 200 mM sodium citrate (pH 3.0, 4.0, 5.0, and 6.0), 200 mM potassium phosphate (pH 6.0, 7.0, and 8.0), and 200 mM NH₄Cl/NaOH (pH 8.0, 9.0, 9.5, and 10.0). For the pH stability studies, the enzyme solution was pre-incubated at room temperature for 2 h with the above-mentioned buffers of different pH (3–10), and for the thermal stability studies, pre-incubated for 30 min with each temperatures from 22 to 70 °C with 5 °C increments. The enzyme activity of the pH studies was assayed at 50 °C for 30min. The activity of the optimum temperature studies was assayed at each temperature. The activity of the thermal stability studies was assayed at 57 °C. Enzyme activity was determined by the initial velocity to produce uric acid, which is detectable at 290 nm, with UV spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan).

Enzyme kinetics

The kinetics parameters, such as Michaelis–Menten constant (K_m) and k_{cat} values against xanthine were calculated from Hanes-Woolf plot. Molar extinction coefficients for xanthine and uric acid at 290 nm were 8.4 and 2.9 [mM⁻¹cm⁻¹], respectively.

Results

Screening of hypoxanthine-degrading microorganisms

Three hundred fifty strains were isolated from soils as hypoxanthine-degrading microorganisms. These strains were evaluated as to hypoxanthine-degrading activity during cultivation (first screening). Thirty-five strains were selected as good hypoxanthine-degrading strains, 21 strains as good xanthine-producing strains. Through the second screening using resting cells, 4 strains isolated from soils were selected as good hypoxanthine-degrading and xanthine-producing strains. In the third screening using cell-free extracts, 4 strains were confirmed to showing the same activity observed in the second screening. As the results of these screenings, A153 was selected as good hypoxanthine-degrading and xanthine-producing strain.

Identification of selected strain

The strain A153 was identified by 16S rDNA analysis. As the results of the analysis, the 16S rDNA sequence of the strain A153 showed 99.6 % homology with that of *Cellulosimicrobium funkei*.

Analysis of purine metabolism by *C. funkei* A153

Purine metabolism was analyzed by the resting cells of *C. funkei* A153. *C. funkei* A153 had strong deaminase activity such as deamination of adenosine and guanine to inosine and xanthine, respectively. Moreover the strain had strong oxidase activity such as oxidation of hypoxanthine and xanthine to xanthine and uric acid, respectively.

Purification of xanthine oxidase from *C. funkei* A153

Through 8 steps, xanthine oxidase was purified approximately 200,000 fold, with a 15.4 % yield, from a soluble cell-free extracts of *C. funkei* A153 (Table 3-1). The purified xanthine oxidase gave three protein bands on SDS-PAGE (Fig. 3-2A). The molecular mass of these proteins were 110,000 (protein-1), 90,000 (protein-2), and 30,000 (protein-3), respectively. They were blotted on PVDF membrane and then the NH₂-terminal amino acid sequences were analyzed by automated Edman degradation. The NH₂-terminal amino acid sequences were identified as follows. (X) means putative amino acid. :

NH₂-terminal of protein-1; (S)(V)(T)PAPHPAP(V)VTID(G)(R)(T)(A)(R)

NH₂-terminal of protein-2; ST(S)GDQGSTSGDQ(G)(T)X (A)(G)

NH₂-terminal of protein-3; MDLGTVTDLVPTADPAFGRE

Purified enzyme solution was treated by lysyl endopeptidase and then internal peptides were purified and their amino acid sequences were analyzed by automated Edman degradation with a pulse liquid phase sequencer. Four internal sequences were identified as follows. (X) means putative amino acid.:

Internal peptide (a); GNLP(R)(P)TGYRAIEDAVLGR(G)

Internal peptide (b); SMSESPFPNPVA PA LANA

Internal peptide (c); VAAGVAGAAPGDXRLVPGGV

Internal peptide (d); IWNTSWGGNVTTSLPAGPM

The homology of these amino acid sequences were searched by BLAST. NH₂-terminal peptide of protein-3 and internal peptide (d) were homologous with xanthine dehydrogenase, aldehyde dehydrogenase, and carbon monoxide dehydrogenase. Internal peptide (a) and (b) were found to exist in the same subunit.

Table 3-1 Purification of xanthine oxidase from *Cellulosimicrobium funkei* strain A153

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
Cell free extracts	467	2.3×10^{-3}	4.9×10^{-6}	100	1
Ammonium sulfate (40-60%)	129	3.0×10^{-3}	2.3×10^{-5}	130	5
Hiprep 16/10 DEAE FF	37.8	n.d.	n.d.	-	-
Mono Q HR 10/10	11.2	2.7×10^{-2}	2.4×10^{-3}	11.8	490
Phenyl Superose HR 5/5	0.83	2.9×10^{-2}	3.5×10^{-2}	12.6	7140
Mono Q HR 5/5	0.45	3.4×10^{-2}	7.6×10^{-2}	14.9	15500
Superdex 200 PC 3.2/30	n.d.	n.d.	n.d.	-	-
Mono Q PC 1.6/5	0.04	3.5×10^{-2}	9.8×10^{-1}	15.4	200000

n.d., not determined

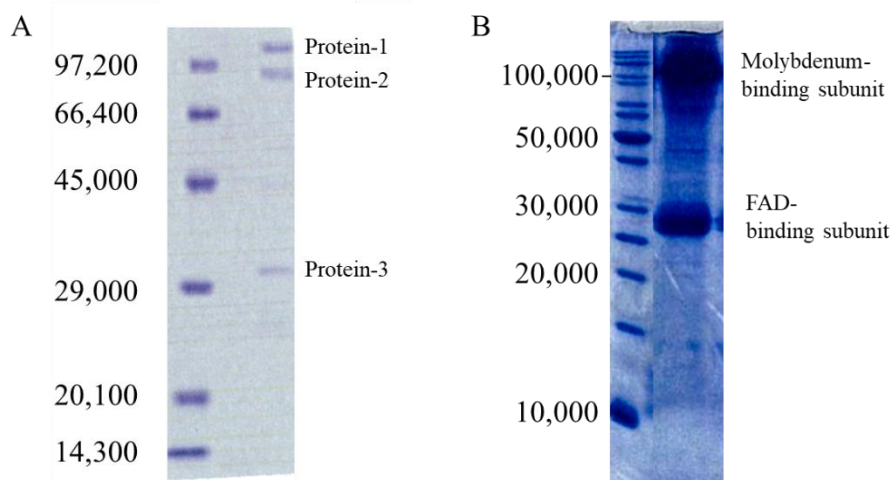


Fig. 3-2 SDS-PAGE of purified xanthine oxidase and recombinant xanthine oxidase.

A, purified xanthine oxidase from *Cellulosimicrobium funkei* strain A153; B, purified his-tagged xanthine oxidase from *Rhodococcus erythropolis* pTipQC1/His-tag-XDH

Genome sequencing

As a result of genome sequencing, it was revealed that the protein-2 was a part of the protein-1, and this enzyme was composed of 2 subunits. The molecular weight of the large subunit was 104,462 and the amino acid sequence was showed about 97 % identity with molybdopterin-dependent oxidoreductase of *Cellulosimicrobium* sp. TH-20 (WP_144680486). The large subunit also had an iron-sulfur cluster-binding domain. The molecular weight of the small subunit was 30,944 and the amino acid sequence was showed about 98 % identity with FAD-binding molybdopterin dehydrogenase of *Cellulosimicrobium* sp. (WP_043659903).

Purification of his-tagged xanthine oxidase

SDS-PAGE of his-tagged xanthine oxidase after purification from *Rhodococcus erythropolis* transformant was shown in Fig. 3-2 B. The molecular weights of large and small subunits were approximately 100,000 and 30,000, respectively.

Activity of the xanthine oxidase with various electron acceptors

Soluble oxygen served as electron acceptor effectively (Table 3-2). The enzyme used various electron acceptors, potassium ferricyanide, 2,6-dichlorophenol-indophenol, phenazine compounds, Methylene blue, NAD⁺, NADP⁺, flavine compounds, and quinone compounds as an electron acceptor.

Table 3-2 Effects of various electron acceptors on the activity of the purified xanthine oxidase

Electron acceptors	Relative activity (%)
Oxygen (dissolved)	100
Potassium ferricyanide	102
2,6-Dichlorophenol indophenols	125
Phenazine methosulfate	109
Methylene blue tetrahydrate	75.5
NAD ⁺	78.1
NADP ⁺	79.7
Riboflavine	69.0
Flavin mononucleotide (FMN)	88.7
Phenazine methyl sulfate (PMS)	122
Phenazine ethyl sulfate (PES)	73.2
α -Naphthoquinone	147
<i>p</i> -Benzoquinone	99.5

The reactions were carried out with 200 μ l of the reaction mixture containing purified enzyme, 20 μ l of 1 mM xanthine, and 50 μ l of 20 mM each electron acceptor at pH 7.0, 45 °C. Enzyme activity was determined by the initial velocity to produce uric acid, which is detectable at 290 nm. They were expressed as relative activity to that with soluble oxygen set as 100 %.

Substrate specificity of the xanthine oxidase

The substrates specificity of the enzyme was investigated. The relative activities of various compounds were summarized in Table 3-3 with the activity to xanthine set as 100 %. Xanthine, hypoxanthine, and purine were good substrate for this enzyme. The enzyme could also oxidize adenine, guanine, isoguanine, and theophylline. However, uric acid, caffeine, theobromine, and pyrimidine could not be confirmed appropriate oxidation products.

Table 3-3 Substrate specificity of xanthine oxidase from *Cellulosimicrobium funkei* strain A153

Substrate		Relative activity (%)	Products*	
Xanthine		100	Uric acid	
Hypoxanthine		27.0	Xanthine	
Adenine		4.5	8-Hydroxyadenine	
Guanine		6.3	8-Oxoguanine	
Isoguanine		8.1	2,8-Dihydroxyadenine	
Purine		15.0	not determined (The product was not hypoxanthine.)	
Theophylline		1.3	1,3-Dimethyluric acid	
Paraxanthine		tr.	1,7-Dimethyluric acid	

Following substrates did not serve as substrates; Uric acid  Theobromine  Caffeine  Pyrimidine

The reactions were carried out with 200 µl of the reaction mixture containing purified enzyme, 1.2 µl of 100 mM 4-AA, 1.4 µl of 100 mM TOOS, 2 µl of 10 mg/ml peroxidase, and 20 µl of 1 mM each substrate, at 7.0, 45 °C. Enzyme activity was determined by the UV spectrum change at 550 nm. The listed products were identified by LC-MS analysis. They were expressed as relative activity to that with xanthine set as 100 %. n.d., not detected, tr., trace amount

Effect of reaction conditions and enzyme stability

Optimal reaction temperature and pH were 55 °C and 7.0, respectively. For pH stability test, the xanthine oxidase kept 80 % of its maximum activity from pH 6.0 to pH 8.0. For thermal stability test, the xanthine oxidase kept 80 % of its maximum activity under 50 °C (Fig. 3-3).

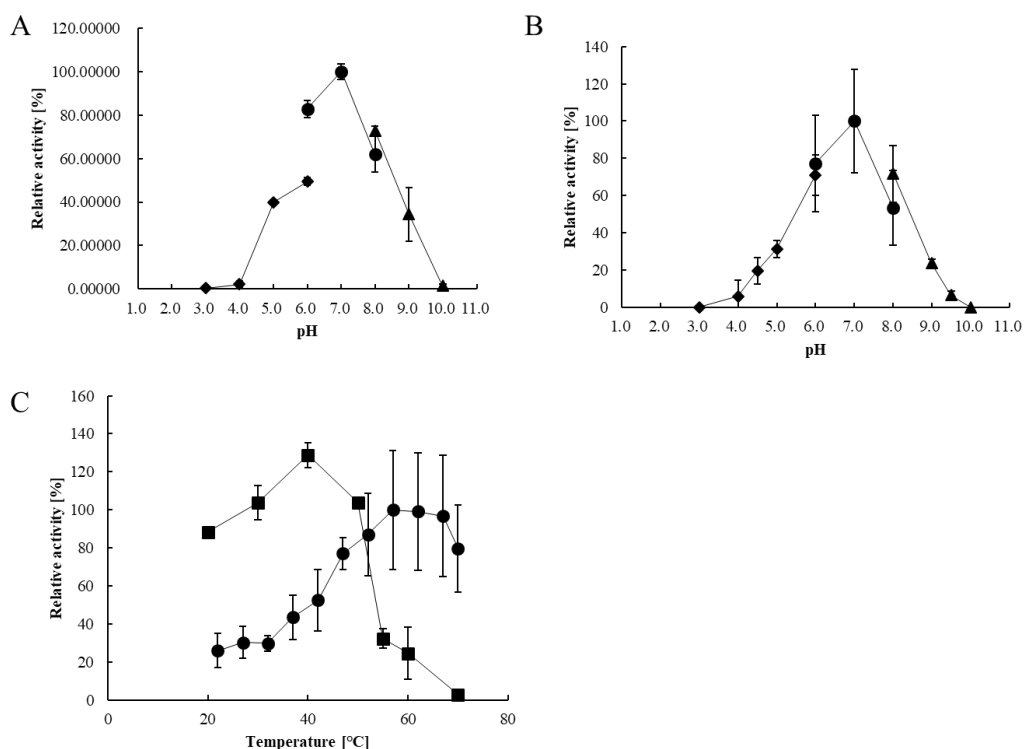


Fig. 3-3 Effects of reaction conditions and enzyme stability

The reactions were carried out with 200 μ l of the reaction mixture containing purified enzyme and 20 μ l of 1 mM xanthine. Enzyme activity was determined by the initial velocity to produce uric acid, which is detectable at 290 nm. A, effect of reaction pH. diamond, sodium citrate buffer; circle, KPB; triangle, $\text{NH}_4\text{Cl}/\text{NaOH}$. The reaction temperature was at 50 °C for 30 min. B, pH stability. diamond, sodium citrate buffer; circle, KPB; triangle, $\text{NH}_4\text{Cl}/\text{NaOH}$. The enzyme solution was pre-incubated for 2 h with buffers of different pH 3.0–10.0. The reaction temperature was at 50 °C for 30 min. C, effect of reaction temperature (circle) and thermal stability (square). For the thermal stability studies, the enzyme solution was pre-incubated at various temperature (22 -70 °C) for 30 min, assayed at 57 °C for 30 min.

Enzyme kinetics

The k_{cat} , K_{m} , and $k_{\text{cat}}/K_{\text{m}}$ values for xanthine were 1.4 sec^{-1} , 0.22 mM, and 6.4 $\text{sec}^{-1}\text{mM}^{-1}$, respectively.

Discussion

In chapter III, the author attempted to design a new prophylaxis for hyperuricemia using enzymes derived from microorganisms. Purine bases were known to have a smaller risk of increasing serum uric acid level than the other purines. On the other hand, hypoxanthine was known to have potency of increasing serum uric acid level in purine bases. Therefore the author attempted the screening of microorganisms transforming hypoxanthine to the other compounds such as xanthine and uric acid which are hard to be adsorbed in intestine. As a result of screening, *Cellulosimicrobium funkei* A153 isolated from soils was selected as good hypoxanthine-degrading microorganisms. Evaluation of the purine metabolism in *C. funkei* A153 revealed that this strain had strong hypoxanthine-oxidizing activity to xanthine and uric acid. The enzyme involved in hypoxanthine degradation in *C. funkei* A153 was purified and characterized. The analysis of protein sequence and genome sequence of *C. funkei* A153 revealed that this enzyme was a xanthine oxidoreductase family protein, which was encoded by a gene cluster consisted of FAD-binding protein and molybdenum-binding protein. Heptapeptide motif, CXXXXCXXC~C, which could interact with iron-sulfur cluster, was located within the N-terminal of molybdenum-binding subunit. There are a few reports of xanthine oxidoreductase like *C. funkei* A153 which have molybdenum-binding subunit containing iron-sulfur cluster-binding domain. Most of the bacterial xanthine oxidoreductase (XOR) family proteins are composed of two subunits, XDHA subunit comprising an N-terminal iron-sulfur domain and a C-terminal FAD domain, and molybdopterin-containing XDHB subunit (42). Mammalian xanthine dehydrogenases are composed of single polypeptide comprising N-terminal iron-sulfur-binding domain, intermediate FAD-binding domain, and C-terminal molybdenum-binding domain. Some of the XOR family members lack the FAD-component, such as aldehyde oxidoreductase from *Desulfovibrio gigas* is composed of a single polypeptide, comprising an N-terminal iron-sulfur binding domain and a C-terminal molybdenum-binding domain like *C. funkei* A153 (41).

The xanthine oxidoreductase in *C. funkei* A153 was purified and characterized using recombinant enzyme. This enzyme could use oxygen as an electron acceptor (Table 3-2). Electrons that are passed to the molybdenum during the oxidation reaction are transferred to FAD via the iron sulfur centers. Finally, oxygen molecule, which is the final electron acceptor, is reduced (38). It suggested that the enzyme was a xanthine oxidase. There are a few reports about bacterial xanthine oxidase. Thermostable xanthine oxidase from *Arthrobacter luteus* has been reported (43), and xanthine oxidase from *Cellulosimicrobium cellulans* has been reported to be expressed heterologously in an actinomycetes *Rhodococcus erythropolis* L-88 (44). Xanthine oxidase can be converted reversibly to xanthine dehydrogenase by oxidation of cysteine residues or irreversibly by limited proteolysis (45). This enzyme could also use NAD^+ and NADP^+ as an electron acceptor about 80% of relative activity compared with oxygen molecule. Whether the enzyme from *C. funkei* A153 also shows such characteristics or not is

further interests for its medicinal use especially in intestine. .

This xanthine oxidase could oxidize only purine compounds such as hypoxanthine, xanthine, guanine, and adenine, but pyrimidines were not (Table 3-3). *N*-Methylated nitrogen-containing heterocyclic compounds were not good substrates of this enzyme because of their steric hindrance around oxidation site. This xanthine oxidase could degrade hypoxanthine to uric acid via xanthine. Xanthine and uric acid are much less harmful than hypoxanthine to the gouty subjects. Therefore it was expected that the enzyme is useful for decreasing serum uric acid level through transforming easily absorbed purine base, hypoxanthine, to hardly absorbed purines, xanthine and uric acid in intestine. And this may be a new prophylaxis for hyperuricemia using enzymes derived from microorganisms.

Summary

Purine base-degrading enzymes are promising as medication and diagnostic tools for hyperuricemia. *Cellulosimicrobium funkei* A153 was found as a good catalyst for hypoxanthine degradation. *C. funkei* A153 could oxidize hypoxanthine to uric acid via xanthine. The enzyme catalyzing this oxidation was purified and characterized. The results of N-terminal and internal amino acid sequences of the purified enzyme and genome sequence analysis indicated that it was found to be homologous with xanthine dehydrogenase, aldehyde dehydrogenase, and carbon monoxide dehydrogenase. This enzyme was consisted of two subunits with molecular weights of about 100,000, molybdenum-binding subunit containing iron-sulfur-binding domain, and 30,000, FAD-binding subunit. This xanthine dehydrogenase family protein in *C. funkei* A153 was cloned, expressed in *Rhodococcus erythropolis* L88, purified as a his-tagged protein, and characterized. This enzyme could use molecular oxygen as an electron acceptor, so it was a xanthine oxidase. The xanthine oxidase could oxidize hypoxanthine, an easily adsorbed purine, to xanthine and further to uric acid, hardly adsorbed purines, and promising as a tool for hyperuricemia medication. This xanthine oxidase was stable under 50 °C and had the maximal activity at pH 7.0. The k_{cat} , K_m , and k_{cat}/K_m values for xanthine were 1.4 sec^{-1} , 0.22 mM, and $6.4 \text{ sec}^{-1}\text{mM}^{-1}$, respectively.

Chapter IV

Screening of purine base-metabolizing probiotic bacteria and evaluation of their effects on purine base-permeability of small intestine with Caco-2 cell co-cultivation assay

In chapter III, only purine metabolizing activity was the evaluation factor for lowering serum uric acid level in microorganisms. However, this evaluation factor was not enough to decide whether or not it could be applied to practically. In order to get much more accurate estimation, the *in vivo* activities for lowering serum uric acid level must be taken into account. However, only a limited number of *in vivo* methods for precisely investigating small intestinal absorption and gut wall excretion have been reported. Further, most studies in human and experimental animals were complex, time- consuming, and expensive, and occasionally require sophisticated equipment.

The Caco-2 cell line, derived from human colon adenocarcinomas, exhibits enterocyte-like characteristics, such as the expression of brush-border enzymes, nutrient transporters, and an intercellular tight junction, and has been used widely as an *in vitro* model for studies of the small intestine, as well as for studying drug metabolism and transport (46-52). Recently, many researchers have utilized these cells with various bacteria to investigate the probiotic function. In general, probiotic function with Caco-2 cell was evaluated by the adhesion competition with pathogens and candidate bacteria or immune response resulted from co-culture (53).

In chapter IV, the author established a novel evaluation system which was focused on the metabolizing or adsorbing activity of probiotic bacteria. Here, the author described the results of the co-culture of probiotic bacteria and Caco-2 cells in the presence of purine compounds. The amounts of purine penetrated into the basal side indicated the ability of the bacteria to lowering serum uric acid level.

Materials and methods

Materials

Purine nucleosides and bases were purchased from WAKO Pure Chemical Industries Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (New York, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Utah, USA). All other chemicals used in this work were of analytical grade and commercially available.

Isolation of hypoxanthine-degrading probiotic bacteria

Lactic acid bacteria isolated from foods were used for following experiments.

First screening

Lactic acid bacteria were inoculated into 15 ml of MRS medium (1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2% K₂HPO₄, 0.5% sodium acetate, 0.2% diammonium citrate, 0.02% MgSO₄·7H₂O, 0.005% MnSO₄·5H₂O, pH 6.5) supplemented with 0.15% (w/v) hypoxanthine and incubated statically at 37°C for 2 days. After the incubation, 400 µl of the each culture broths were diluted 3 times by adding DMSO. Then the supernatants were analyzed by HPLC as described below.

Second screening

Selected lactic acid bacteria with hypoxanthine-degrading activity in the 1st screening were used. Reaction mixture (500 µl) comprising 25 mM hypoxanthine, 200 mM buffer (AcONa-HCl; pH 4.5, Tris-HCl; pH 9.5, or none; pH 8.0), 0.4% (v/v) polyoxyethylenelaurylamine, 1% (v/v) xylene, 5 mM MgSO₄ · 7H₂O, and 50% (w/v) washed wet cells was added into an eppendorf tube and incubated at 37°C with shaking (300 strokes/min) for 2 days. After reaction, the reaction mixtures were diluted 3 times by adding DMSO and centrifuged at 10,000 rpm for 10 min (LC-100). The supernatants were analyzed by HPLC.

Third screening

Hypoxanthine-degrading activity of the selected strains in the 2nd screening were evaluated using cell-free extracts (CFEs) disrupted by sonication. Reaction mixture (200 µl) comprising 0.5 mM hypoxanthine (in 25 mM NaOH), 200 mM Tris-HCl buffer (pH 7.4) and 1.25% (v/v) CFEs was added into an eppendorf tube and incubated at 37°C with shaking (150 shakes/min) for 10 min. After the reaction, the reaction mixtures were centrifuged at 10,000 rpm for 10 min and the supernatants were analyzed by HPLC.

HPLC analysis

Quantitative analysis of hypoxanthine-degradation was performed by reverse-phase high-performance liquid chromatography (HPLC) by using a Shimadzu LC-VP system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C18-PAQ column (250 x 4.6 mm, Nacalai tesque Co.,Ltd, Japan). The mobile phase was 50 mM K₂HPO₄ (pH 2.0) at a flow rate of 1.0 ml/min and the effluents were monitored by UV detection at 254 nm. Ten µl of the reaction mixture was injected directly.

Identification of selected strains

After the three stages of screening, one strain isolated from soils and one lactic acid bacterium kept in the author's laboratory were selected, and they were identified by TechnoSuruga Laboratory Co., Ltd based on 16S rDNA analysis.

Analysis of purine metabolism

Purine-decomposing activity was evaluated by using resting cells. Reaction mixture (300 μ l) comprising 1 mM purine (in 25 mM NaOH), 200 mM buffer (Tris-HCl; pH 7.4), 0.1% (v/v) polyoxyethylenelaurylamine, 0.3% (v/v) xylene, 1.7 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1% (w/v) washed wet cells was added to an eppendorf tube and incubated at 37°C with shaking (150 strokes/min) for 24 h. After the reaction, the reaction mixtures centrifuged at 10,000 rpm for 10 min. The supernatants were analyzed by HPLC.

Caco-2 cell cultures

Caco-2 cells were obtained from the American Type Culture Collection (Virginia, USA). The Caco-2 cells were maintained in DMEM containing 300 ng/ml L-glutamine and supplemented with 10 % FBS, 100 U/ml penicillin, and 100 ng/ml streptomycin. The cells were incubated under a humidified atmosphere of 95 % O_2 and 5 % CO_2 at 37° C, and maintained at 80-90 % confluence.

Caco-2 cells were seeded on cell culture inserts (PET track-etched membrane, 23 mm in diameter, pore size 0.4 μm , Becton Dickinson Labware, New Jersey, USA) in 6-well plates at a density of 8×10^4 cells/insert. The apical and basal compartments contained 2.0 and 3.0 ml of culture medium, respectively. Medium was replaced freshly every three days. The cell culture inserts were used for the experiments 21-30 days after seeding, when TER (transepithelial electrical resistance) value exceeded 300 Ω .

Bacterial strains

Lactobacillus paracasei N103 was cultured in 15 ml of MRS broth with standing at 37°C for 12-16 h. After cultivation, 1 ml of culture broth was collected and washed with potassium phosphate buffer (PBS), pH 7.2, 2 times. Then it was diluted with DMEM containing 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (FBS-free, antibiotics-free) medium to final OD_{600} of 0.006. This diluted suspension was used for co-culture with Caco-2 cells, and, as a control, bacterial strain-free DMEM containing 25 mM HEPES (FBS-free, antibiotics-free) medium was used.

Measurement of TER

The TER value of the Caco-2 monolayers was measured using a Millicell-ERS instrument (MILLIPORE, Massachusetts, USA). The measurements were performed prior to and at the end of each experiment to assess the integrity of Caco-2 monolayers. Monolayer resistance, obtained by subtracting intrinsic resistance (membrane alone) from total resistance (membrane plus monolayer), was corrected for the surface area and expressed as Ωcm^2 . In all the experiment, it was decided that the surface area of the cell culture insert for 6-well plates was 4.2 cm^2 and intrinsic resistance was 160 Ω .

MTT assay

The cell toxicity of purines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. MTT, which was yellow tetrazolium salt, was reduced in metabolically active cells to form insoluble purple formazan crystals, which were solubilized by the addition of a detergent. The color could then be quantified by spectrophotometric means.

The end of each experiment, apical and basal both sides of medium were removed and washed with PBS buffer. Cell culture inserts were transferred to new plates. Both sides were filled with 1.8 ml (for apical side) and 3.0 ml (for basal side) of phenol red-free DMEM medium and added 200 μ l of 5 mg/ml MTT (in PBS) and then incubated at 37°C for 1 h. After incubation, both sides of medium were removed and 2 ml of DMSO/2-propanol (1:1) was added to each insert to dissolve formazan. The solutions were centrifuged at 10,000 rpm for 10 min and then absorbance of the supernatants was measured at 530 nm and 630 nm as a background.

Purine penetration assay

After Caco-2 cells were differentiated, purine penetration was evaluated. Both sides of medium were removed and washed with PBS buffer twice and then filled with 1.8 ml (for apical side) and 3.0 ml (for basal side) of DMEM medium. Two hundred μ l of 2 mM purine (in 25 mM NaOH) was added into apical side and incubated at 37 °C for 1 day with 5 % CO₂. One hundred μ l of both sides medium were collected hourly and added the equal amount of DMEM medium. Then the collected mediums were centrifuged at 10,000 rpm for 5 min and the supernatants were analyzed by HPLC. The viability of cells was evaluated by MTT assay.

Purine penetration assay with *L. paracasei* N103

After Caco-2 cells were differentiated, both sides of medium were removed and washed with PBS buffer twice and then filled with 0.8 ml (for apical side) and 3.0 ml (for basal side) of antibiotics free-DMEM containing 25 mM HEPES and 10 % FBS medium. Then 1 ml of *L. paracasei* N103 suspension described above was added into apical side and incubated at 37 °C for 3-5 h with 5 % CO₂ to stabilize Caco-2 monolayers. After stabilization, Two hundred μ l of 2 mM purine (in 25 mM NaOH) was added into apical side and incubated at 37 °C for 3 days with 5 % CO₂. One hundred μ l of both sides medium were collected hourly and added the equal amount of DMEM medium. Then the collected mediums were centrifuged at 10,000 rpm for 5 min and the supernatants were analyzed by HPLC. The integrity of Caco-2 monolayer was evaluated the value of TER.

Results

Screening of hypoxanthine-degrading probiotics

One hundred fifty lactic acid bacteria were evaluated as to their hypoxanthine-degrading activity during cultivation (first screening), and then 19 strains were selected as unknown compounds-producing strains. Through the second screening using resting cells, one lactic acid bacterium strain N103 was selected as an unknown compounds-producing strain. Although the strain N103 did not show the same activity observed in the second screening in the third screening using cell-free extracts, tN103 was selected as an unknown compounds-producing strain from hypoxanthine.

Purine penetration assay by Caco-2 monolayer

The fate of purines added to apical side of Caco-2 monolayers were investigated as to how they were metabolized and accumulated in both apical and basal sides. On MTT analysis, the values of absorbance 530 nm-630 nm of each purine supplemented samples were not much different from the control without purines; indicated that purines used in these experiments were not harmful for Caco-2 cells. The following results were shown in Fig. 4-1.

i) Adenosine addition

Adenosine was metabolized to inosine rapidly and then inosine was gradually metabolized to hypoxanthine and finally accumulated mainly in the form of hypoxanthine and partly xanthine in both apical and basal sides. Moreover, uric acid was accumulated in basal side.

ii) Inosine addition

Inosine was gradually metabolized to hypoxanthine and accumulated mainly in the form of hypoxanthine and partly xanthine in both apical and basal sides. Moreover, uric acid was accumulated in basal side.

iii) Xanthosine addition

Xanthosine was partly metabolized to xanthine and accumulated mainly in the form of xanthosine and partly xanthine in apical side. Moreover, uric acid was accumulated in basal side.

iv) Guanosine addition

Guanosine was gradually metabolized to guanine and then guanine was rapidly metabolized to xanthine and finally accumulated in the form of xanthine in both apical and basal sides. Moreover, uric acid was accumulated in basal side even in 20 h.

v) Adenine addition

Adenine was gradually metabolized to hypoxanthine and then hypoxanthine was gradually metabolized to xanthine and accumulated mainly in the form of adenine, and partly hypoxanthine and xanthine in both apical and basal sides. Moreover, uric acid was accumulated in basal side.

vi) Hypoxanthine addition

Hypoxanthine was slowly metabolized to xanthine and accumulated mainly in the form of hypoxanthine and partly xanthine in both apical and basal sides. Moreover, uric acid was accumulated in basal side.

vii) Xanthine addition

Xanthine was not metabolized to anything and accumulated in the form of xanthine in both apical and basal sides. However, uric acid was accumulated in basal side.

viii) Guanine addition

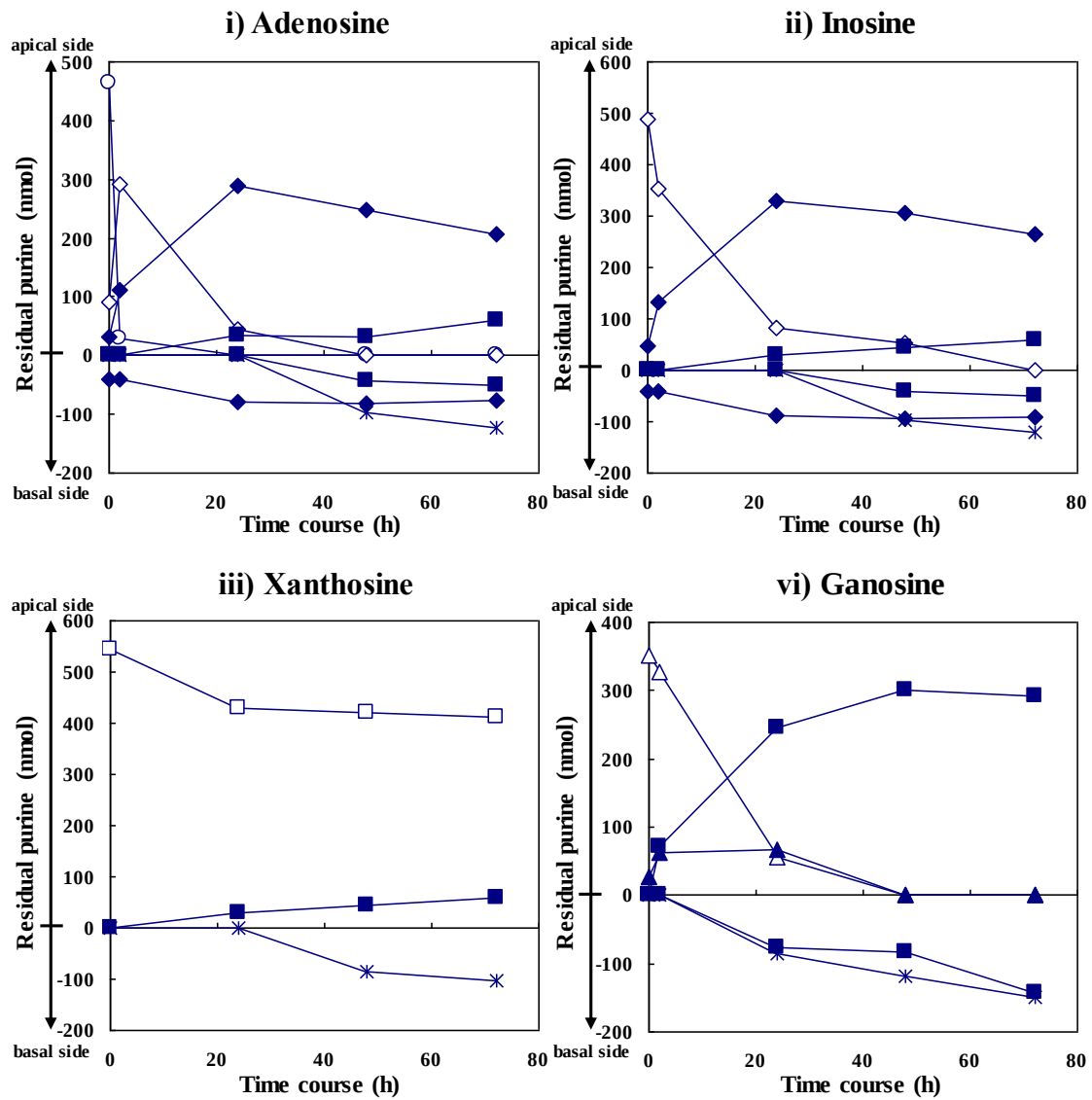
Guanine was gradually metabolized to xanthine and accumulated in the form of xanthine in both sides. Moreover, uric acid was accumulated in basal side even in 20 h.

ix) Uric acid addition

Uric acid was not metabolized to anything and accumulated in the form of uric acid in basal side even in 20 h.

x) No addition

Though there was no purine addition, uric acid was accumulated in basal side. Considering this, part of the uric acid observed might be exogenous one.



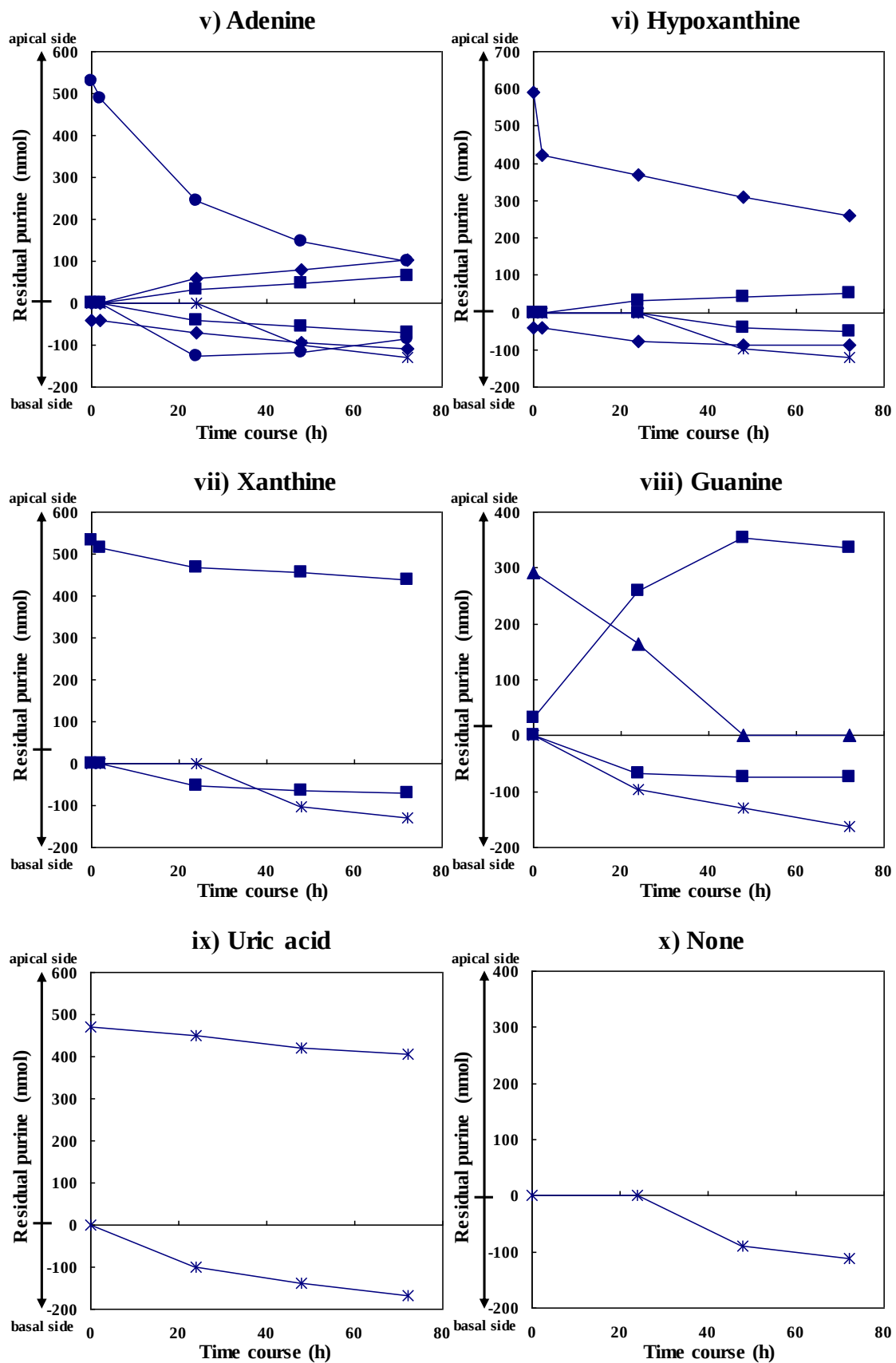


Fig. 4-1 Time course of purine degradation by Caco-2 monolayer

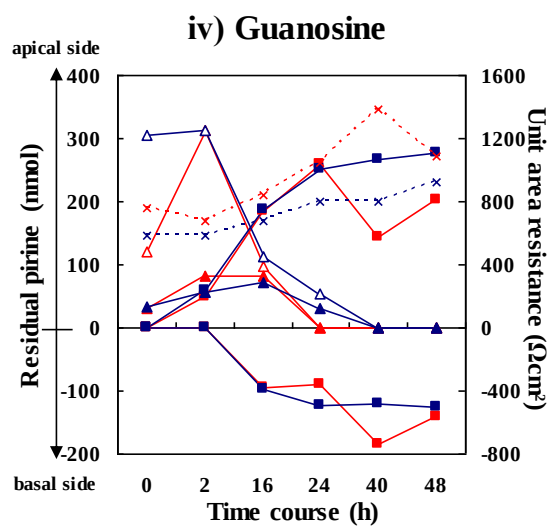
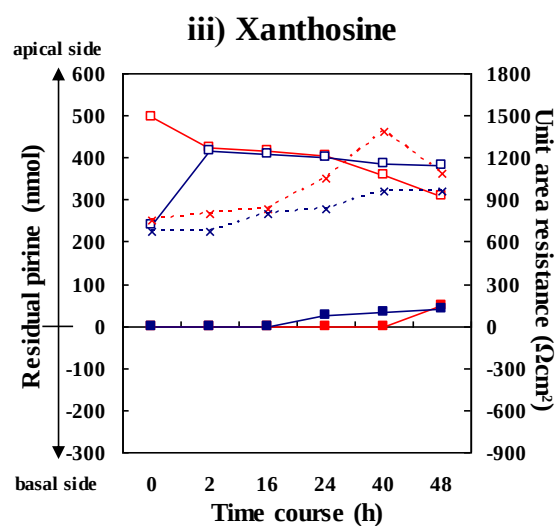
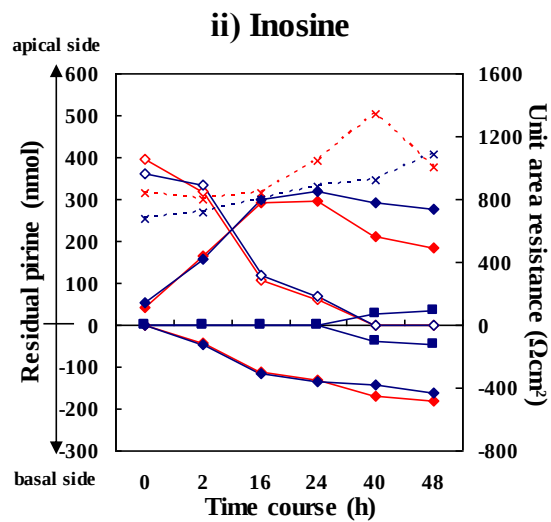
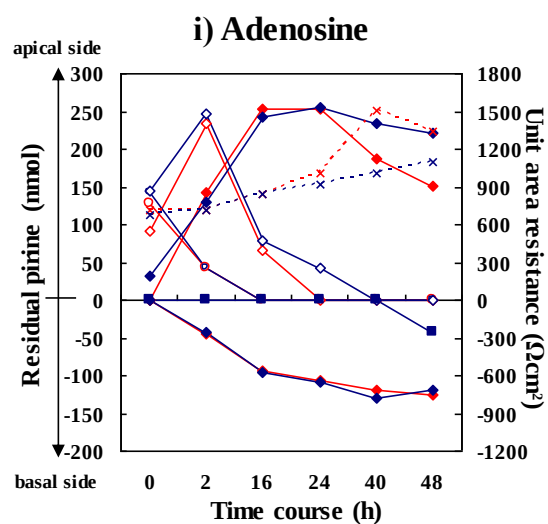
Residual purines (i; adenosine, ii; inosine, iii; xanthosine, iv; guanosine, v; adenine, vi; hypoxanthine, vii; xanthine, viii; guanine, ix; uric acid, x; none) in both apical and basal sides. Adenosine; open circle, Inosine; open diamond, Xanthosine; open square, Guanosine; open triangle, Adenine; closed circle, Hypoxanthine; closed diamond, Xanthine; closed square, guanine; closed triangle, Uric acid; asterisk.

Purine penetration assay with *L. paracasei* N103

The effects of *L. paracasei* N103 on purine penetration in co-cultivation with Caco-2 monolayer were investigated. The accumulation of lactic acid in the cell culture insert through lactic acid fermentation by *L. paracasei* N103 decreased the pH and disrupted the monolayer. To evaluate the amount of purines which were penetrated into basal side, the Caco-2 monolayer had to be maintained at least 48 h which was enough to confirm purine penetration clearly. To maintain the medium pH, the DMEM containing 25 mM HEPES medium was used. And to control the growth of the bacteria for repressing lactic acid fermentation, the seed bacterial solution was diluted to OD600 at 0.006 with DMEM containing 25 mM HEPES medium.

Under the condition stated above, purine penetration assay with *L. paracasei* N103 was performed (Fig. 4-2). Adenosine, inosine, guanosine, and guanine were able to be metabolized and accumulated in the forms of hypoxanthine, hypoxanthine, xanthine, and xanthine, respectively. Focused on adenosine and inosine metabolism, the final product was hypoxanthine but not xanthine which was the final product only with Caco-2 monolayer. On the other hand, focused on xanthosine, guanosine, and guanine metabolism, the final product was xanthine. Moreover, in the case of xanthine addition, xanthine was not metabolized but only penetrated into basal side. These facts suggested that the addition of *L. paracasei* N103 inhibited the accumulation of xanthine which was derived from hypoxanthine. There was a possibility that the hypoxanthine-transforming activity to xanthine in or on the surface of Caco-2 monolayer was inhibited by *L. paracasei* N103.

In addition, the more *L. paracasei* N103 grew, the higher unit area resistance was. It suggested that *L. paracasei* N103 was able to develop the tight junction of Caco-2 monolayer.



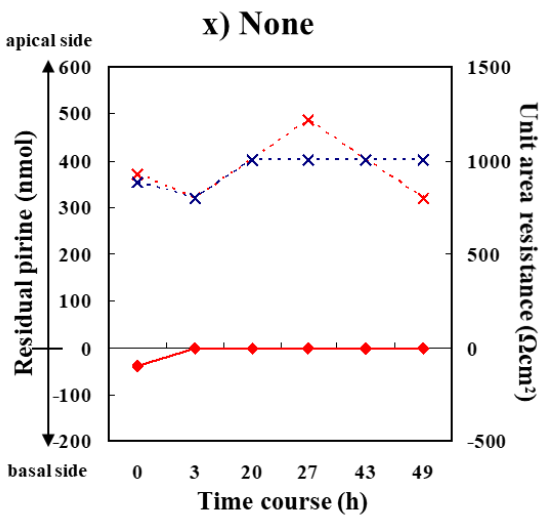
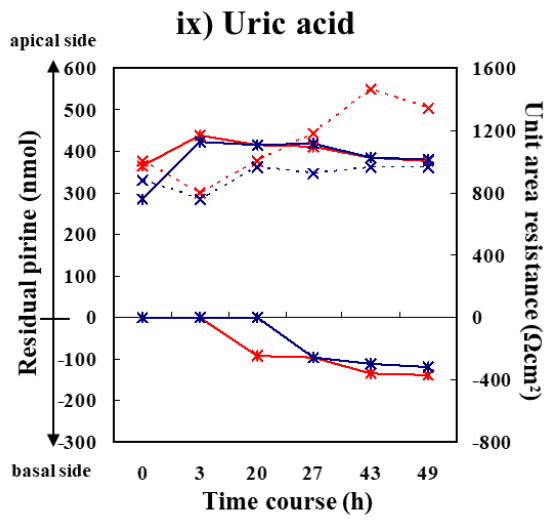
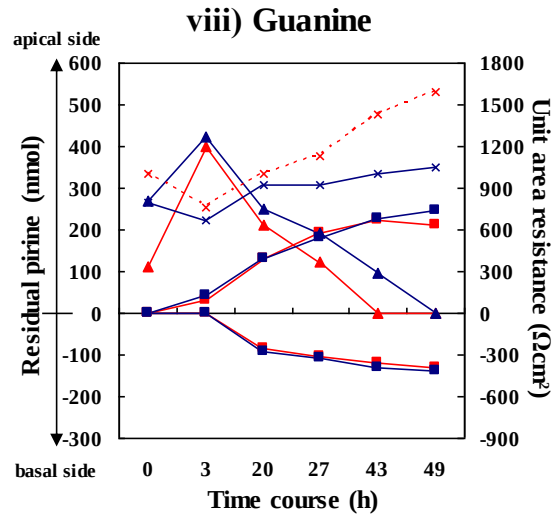
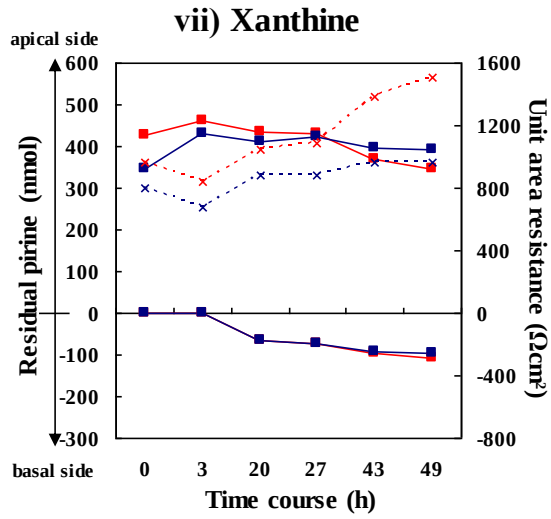
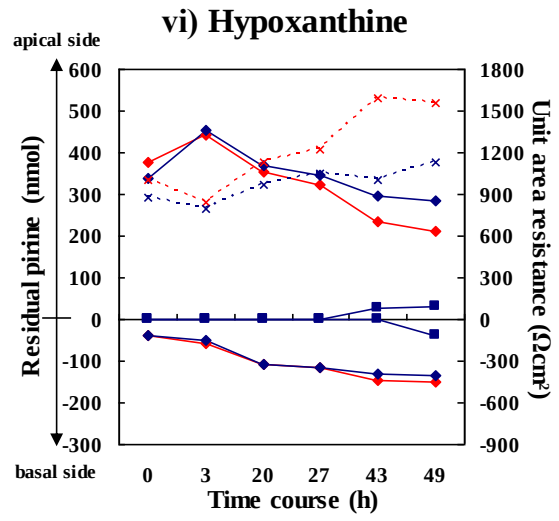
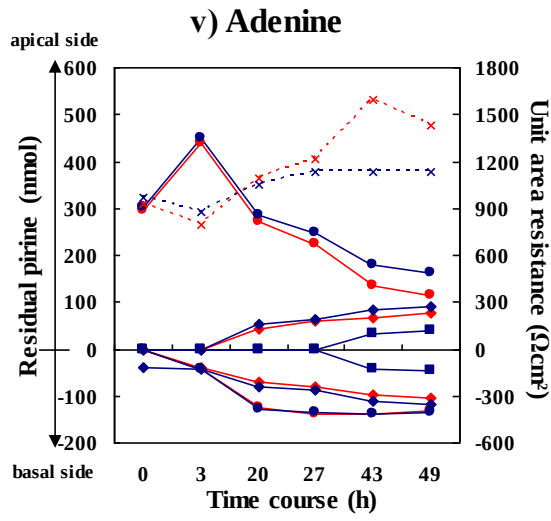


Fig. 4-2 Time course of purine degradation by Caco-2 monolayer with or without *L. paracasei* N103.

Residual purines (i; adenosine, ii; inosine, iii; xanthosine, iv; guanosine, v; adenine, vi; hypoxanthine, vii; xanthine, viii; guanine, ix; uric acid, x; none) in both apical and basal sides. Red; with *L. paracasei* N103, Blue; without *L. paracasei* N103, Dashed line; TER, Adenosine; open circle, Inosine; open diamond, Xanthosine; open square, Guanosine; open triangle, Adenine; closed circle, Hypoxanthine; closed diamond, Xanthine; closed square, guanine; closed triangle, Uric acid; asterisk.

Discussion

The author attempted to design a new assay system using Caco-2 monolayer for evaluating whether probiotics had the ability of lowering serum uric acid level. For designing, there were three problems to check, cell toxicity of purines, penetration of purines by Caco-2 monolayer, and effects of co-culture of probiotic bacteria and Caco-2 cells.

Various purines were added to the differentiated Caco-2 cell monolayer to evaluate purine metabolism and penetration. After incubation with purines, MTT assay was performed to check the cell toxicity of purines. The values of absorbance 530 nm-630 nm in the MTT assay were not much different from that of the control which was not added purines. It suggested that 0.2 mM purines in final concentration were not showed the cell toxicity. The 0.2 mM purines were analyzable by HPLC therefore purine penetration was evaluated by the amount of purines in the basal side of Caco-2 monolayer after incubation with 0.2 mM purines in the apical side.

Focused on purine metabolism of Caco-2 monolayers, adenosine, Inosine, guanosine, and guanine were able to be rapidly metabolized. Moreover, purine nucleosides and purine bases were seemed to be metabolized to xanthine and accumulated in apical side. As to penetration to basal side, adenine, hypoxanthine, xanthine, and uric acid were able to be penetrated into basal side. The investigation whether probiotic bacteria were able to lower the amount of purines which were penetrated into basal side might suggest the ability of lowering serum uric acid level.

As a result of co-culture with *L. paracasei* N103 and Caco-2 cells, the tight junction of Caco-2 monolayer was developed and it suggested that *L. paracasei* N103 had the ability of increasing barrier function of the intestine. The amount of purines penetrated into basal side was not so much different from with or without *L. paracasei* N103. It suggested that the effects of *L. paracasei* N103 on lowering serum purine level is not so significant. However, the amounts of inosine, xanthosine, and guanosine in the apical side decreased faster than that without *L. paracasei* N103. Moreover, as to the amounts of adenine, hypoxanthine, xanthine and guanine in apical side, they were clearly lower with *L. paracasei* N103 than without *L. paracasei* N103. It suggested that there was a possibility that these purines were accumulated or adsorbed in or on the surface of *L. paracasei* N103. Therefore *L. paracasei* N103 might have a positive

effect on lowering purine level in intestine. Decreasing the amount of purines in intestine by adsorbing purine bases might result in lowering the amount of purines absorbed into blood. There was possibility that *L. paracasei* N103 had the ability of lowering serum uric acid level.

In chapter IV, the author attempted to design of novel evaluation system for purine base-permeability of small intestine with Caco-2 cell co-cultivation assay. *L. paracasei* N103 was tested and there was possibility that it had the ability of lowering serum uric acid level. The author expected that the other probiotic bacteria and purine-degrading enzymes, such as xanthine oxidase found in chapter III, were assayed with this system and ideal probiotics or enzymes for hyperuricemia prevention was discovered.

Summary

A screening system for the ability of lowering serum uric acid level by using differentiated Caco-2 cells as a model of human intestinal epithelial cells was established. At first, purine penetration into Caco-2 cell was checked. Various purines were added to the apical side of differentiated Caco-2 monolayers. After incubation, the both sides of medium were collected and analyzed with HPLC. The result of this analysis suggested that Caco-2 cells could degrade purine nucleosides and purine bases to xanthine and penetrated as forms of adenine, hypoxanthine, xanthine, and uric acid into basal side. Based on these observations, the assay system to evaluate the ability for lowering purine penetration into basal side was designed. *Lactobacillus paracasei* N103 was used for co-culture system with Caco-2 cells. *L. paracasei* N103 suspension was conditioned not to disrupt Caco-2 monolayer and added to the apical side and then incubated a few hours to stabilize the monolayer. After stabilization, various purines were added to apical side and then incubated. The both sides of medium were collected hourly and analyzed with HPLC. As results of these analyses, there were some promising results. The first, *L. paracasei* N103 was able to develop the tight junction of Caco-2 monolayer. The second, there was possibility that *L. paracasei* N103 had inhibition activity of xanthine production from hypoxanthine. Finally, there was possibility that inosine, xanthosine, guanosine, adenine, hypoxanthine, xanthine, and guanine were accumulated or trapped in or on the surface of *L. paracasei* N103. These results suggested that *L. paracasei* N103 might have the ability of lowering serum uric acid level for hyperuricemia prevention.

Conclusions

Chapter I

Microbial enzymes were screened for hydroxylating activity towards the 2-methyl group in a maltol derivative, 2-methyl-3-(phenylmethoxy)-4*H*-pyran-4-one (BMAL). *Novosphingobium* sp. SB32149 was found to have the ability to convert BMAL into 2-(hydroxymethyl)-3-(phenylmethoxy)-4*H*-pyran-4-one (BMAL-OH). The enzymes responsible, a cytochrome P450 monooxygenase (P450nov), a ferredoxin (FDXnov), and a ferredoxin reductase (FDRnov), were identified in the SB32149 strain. In the reaction with recombinant *E. coli* expressing P450nov, FDXnov, and FDRnov, BMAL-OH was successfully produced from BMAL. Moreover, using the directed evolution approach, four amino acid substitutions, L188P/F218L/L237M in P450nov and A10T in FDXnov, were found to enhance BMAL-OH production. Consequently, up to 5.2 g/L BMAL-OH was obtained from 8.0 g/L BMAL by bioconversion using a 250 mL jar fermenter.

Chapter II

Microbial enzymes useful for BMAL-OH oxidation to 3-benzoyloxy-4-oxo-4*H*-pyran-2-carboxylic acid (BMAL-COOH) via 3-benzoyloxy-4-oxo-4*H*-pyran-2-carboxaldehyde (BMAL-CHO) were screened. *Pseudomonas nitroreducens* SB32154 was found to have the ability to convert BMAL-CHO to BMAL-COOH. The enzyme responsible for the aldehyde oxidation, a BMAL-CHO dehydrogenase, was purified from *P. nitroreducens* SB32154 and characterized. The purified BMAL-CHO dehydrogenase was found to be a xanthine dehydrogenase (XDH) family enzyme with unique structure of hetero dimer which was composed of 75 kDa and 15 kDa subunits containing a molybdenum-cofactor and [Fe-S] clusters, respectively. The enzyme showed broad substrate specificity towards benzaldehyde derivatives but was not able to oxidize purine bases, xanthine, and hypoxanthine. The K_m and V_{max} for BMAL-CHO were 24.97 mM and 0.36 U/mg of protein, respectively. Furthermore, one-pot conversion of BMAL to BMAL-COOH via BMAL-CHO by combined action of the BMAL-CHO dehydrogenase with P450nov was achieved.

Chapter III

Purine base-degrading enzymes are promising as medication and diagnostic tools for hyperuricemia. *Cellulosimicrobium funkei* A153 was found as a good catalyst for hypoxanthine degradation. *C. funkei* A153 could oxidize hypoxanthine to uric acid via xanthine. The enzyme catalyzing this oxidation was purified and characterized. The results of N-terminal and internal amino acid sequences of the purified enzyme and genome sequence analysis indicated that it was found to be homologous with xanthine dehydrogenase, aldehyde dehydrogenase, and carbon monoxide dehydrogenase. This enzyme was consisted of two subunits with molecular weights of about 100,000, molybdenum-binding subunit containing iron-sulfur-binding domain, and 30,000, FAD-binding subunit. This xanthine dehydrogenase family protein in *C. funkei* A153 was cloned, expressed in *Rhodococcus erythropolis* L88, purified as a his-tagged protein, and characterized. This enzyme could use molecular oxygen as an electron acceptor, so it was a xanthine oxidase. The xanthine oxidase could oxidize hypoxanthine, an easily adsorbed purine, to xanthine and further to uric acid, hardly adsorbed purines, and promising as a tool for hyperuricemia medication. This xanthine oxidase was stable under 50 °C and had the maximal activity at pH 7.0. The k_{cat} , K_m , and k_{cat}/K_m values for xanthine were 1.4 sec^{-1} , 0.22 mM, and $6.4 \text{ sec}^{-1}\text{mM}^{-1}$, respectively.

Chapter IV

A screening system for the ability of lowering serum uric acid level by using differentiated Caco-2 cells as a model of human intestinal epithelial cells was established. At first, purine penetration into Caco-2 cell was checked. Various purines were added to the apical side of differentiated Caco-2 monolayers. After incubation, the both sides of medium were collected and analyzed with HPLC. The result of this analysis suggested that Caco-2 cells could degrade purine nucleosides and purine bases to xanthine and penetrated as forms of adenine, hypoxanthine, xanthine, and uric acid into basal side. Based on these observations, the assay system to evaluate the ability for lowering purine penetration into basal side was designed. *Lactobacillus paracasei* N103 was used for co-culture system with Caco-2 cells. *L. paracasei* N103 suspension was conditioned not to disrupt Caco-2 monolayer and added to the apical side and then incubated a few hours to stabilize the monolayer. After stabilization, various purines were added to apical side and then incubated. The both sides of medium were collected hourly and analyzed with HPLC. As results of these analyses, there were some promising results. The first, *L. paracasei* N103 was able to develop the tight junction of Caco-2 monolayer. The second, there was possibility that *L. paracasei* N103 had inhibition activity of xanthine production from hypoxanthine. Finally, there was possibility that inosine, xanthosine, guanosine, adenine, hypoxanthine, xanthine, and guanine were accumulated or trapped in or on the surface of *L. paracasei* N103. These results suggested that *L. paracasei* N103 might have the ability of lowering serum uric acid level for hyperuricemia prevention.

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PUBLICATIONS

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