Full Paper

Isolation of lactic acid bacteria exhibiting high scavenging activity for environmental hydrogen peroxide from fermented foods and its two scavenging enzymes for hydrogen peroxide

(Received October 10, 2015; Accepted November 29, 2015)

Akio Watanabe,^{1,2} Chiaki Kaneko,¹ Yasuhiro Hamada,¹ Kouji Takeda,¹ Shinya Kimata,¹ Takashi Matsumoto,³ Akira Abe,⁴ Naoto Tanaka,⁵ Sanae Okada,⁵ Masataka Uchino,⁶ Junichi Satoh,¹ Junichi Nakagawa,⁷ and Youichi Niimura^{1,*}

¹ Department of Bio-Science, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

² TAIYO Corporation, Laboratory, Higashiyodogawa-ku, Osaka 533-0023, Japan

³ Nodai Genome Research Center, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

⁴ Department of Ophthalmology, Sapporo Medical University, Hokkaido 060-8556, Japan

⁵ Nodai Culture Collection Center, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

⁶ Department of Applied Biology and Chemistry, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

⁷ Department of Food Science and Technology, Tokyo University of Agriculture, Abashiri-shi, Hokkaido 099-2493, Japan

To obtain lactic acid bacteria that scavenge environmental hydrogen peroxide, we developed a specialized enrichment medium and successfully isolated *Pediococcus pentosaceus* Be1 strain from a fermented food. This strain showed vigorous environmental hydrogen peroxide scavenging activity over a wide range of hydrogen peroxide concentrations. High Mn-catalase and NADH peroxidase activities were found in the cell-free extract of the *P. pentosaceus* Be1 strain, and these two hydrogen peroxide scavenging enzymes were purified from the cell-free extract of the strain. Mn-catalase has been purified from several microorganisms by several researchers, and the NADH peroxidase was first purified from the original strain in this report. After cloning the genes of the Mn-catalase and the NADH peroxidase, the deduced amino acid sequences were compared with those of known related enzymes.

Key Words: bacterial living cells; hydrogen peroxide; lactic acid bacteria; NADH peroxidase; scavenging of hydrogen peroxide

Introduction

Lactic acid bacteria have not been recognized as potent hydrogen peroxide scavengers, because some strains are known to accumulate hydrogen peroxide in the culture medium (Endo et al., 2009; Murphy and Condon, 1984; Sakamoto and Komagata, 1996; Whittenbury, 1964). However, there have been some reports regarding various hydrogen peroxide-degrading intracellular enzymes of lactic acid bacteria (Dolin, 1953; Hanson and Haggstrom, 1984; Mochizuki et al., 2012). *Streptococcus mutans*, *Lactococcus lactis*, and *S. agalactiae* have a two-component peroxide-scavenging system that consists of AhpF (or NADH oxidase) and AhpC (Prx) (Jiang et al., 2005; Lechardeur et al., 2010; Poole et al., 2000). AhpF and the NADH oxidase belong to the peroxiredoxin reductase family and function as peroxide scavengers, showing an extremely fast reaction rate in spore-forming lactic acid bacteria and related species, such as *Sporolactobacillus inulinus* (Nishiyama et al., 1997) and *Amphibacillus xylanus* (Niimura and Massey, 1996; Niimura et al., 1995), which lack a respiratory chain and heme-catalase as do lactic acid bacteria. Accordingly, some lactic acid bacteria must have a highly active hydrogen peroxide scavenging enzyme (Jiang et al., 2005; Lechardeur et al., 2010;

*Corresponding author: Youichi Niimura, Department of Bio-Science, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan.
E-mail: niimura@nodai.ac.jp

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Isolation of lactic acid bacteria exhibiting high scavenging activity for environmental hydrogen peroxide from fermented food.

	Sources for screening			Total number
	Rice malts	Fermented vegetables with salts	Salted radish aged with koji rice	
Number of sources for screening	5	22	1	28
Isolates in 1st isolating step	255	76	239	570
Isolates after 5th isolating steps with enrichment	32	16	18	66
Isolates selected by H ₂ O ₂ scavenging activity	14	0	3	17

Poole et al., 2000), and might exhibit high scavenging activity for environmental hydrogen peroxide. However, we could not find any lactic acid bacteria exhibiting a high scavenging activity for environmental hydrogen peroxide although they have been reported to have a low activity (Talwalkar and Kailasapathy, 2003). In this paper, we report the isolation and characterization of lactic acid bacteria that exhibits a high scavenging activity for environmental hydrogen peroxide.

Materials and Methods

Bacteria and cultures. In an attempt to develop an enrichment medium for screening bacteria exhibiting high scavenging reactive oxygen species (ROS), we added supplements to a basic glucose yeast peptone (GYP) medium (Uchimura and Okada, 1992a). Sources for Fenton reaction-like ROS production: 2.5 mM iron (III) citrate n-hydrate, 2.5 mM iron (II) fumarate. 1% linoleic acid hydroperoxide solution (v/v) suspended in 5% sterilized Tween 80 solution. Inhibitor for growth of aerobic microorganisms: 30 ppm (46 μ M) sodium azide, 30 ppm (10 μ M) cycloheximide. Sources of screening: fermented foods in Table 1. Tested strains (except for *Pediococcus pentosaceus* ATCC 25745 [genome sequencing strain]) were selected in the type strain of lactic acid bacteria. The selected strains and their culture conditions were as follows. *Pediococcus pentosaceus* Be1 strain, *P. pentosaceus* ATCC 25745, *Pediococcus pentosaceus* NRIC 0099^T, *Lactobacillus plantarum* NRIC 1067^T, and the 66 strains of isolates (Table 1), were cultured aerobically in a shaking culture at 37°C in GYP medium. Because *Lactobacillus acidophilus* NRIC 1547^T, *Lactobacillus brevis* NRIC 1638^T, *Lactobacillus casei* NRIC 0644^T, *Lactobacillus delbrueckii* subsp. *bulgaricus* NRIC1688^T, *Lactobacillus delbrueckii* subsp. *delbrueckii* NRIC 0665^T, *Lactobacillus fermentum* NRIC 1752^T, and *Streptococcus thermophilus* NRIC 0256^T were found to be difficult to grow in an aerobic culture, they were grown in a static culture at 37°C in GYP medium.

Assay of hydrogen peroxide scavenging activity for living cells, cell-free extract, and enzymes. The bacterial cells were harvested at their late logarithmic or early stationary growth phase by centrifugation, and washed with 50 mM sodium phosphate buffer (pH 7.0). Their late logarithmic or early stationary growth phase was determined by turbidity at 660 nm. After the turbidity of the washed cells was adjusted to 1.6 optical density at 660 nm, the bacterial cell suspension was used for the determination

of the dry cell weight and measurement of hydrogen peroxide scavenging activity. For the determination of the dry cell weight, 200 ml of the bacterial cell suspension was transferred to a centrifugation tube, and centrifuged at $48,400 \times g_{\max}$ (Avanti HP-25 centrifuge, Beckman) for 10 min. The harvested bacterial cells were dried at 100°C until a constant weight was achieved. To measure the hydrogen peroxide scavenging activity, the remaining bacterial suspension (470 μ l) was diluted four times with the phosphate buffer, and transferred into a 2.0 ml Eppendorf tube. The reaction mixture for the scavenging reaction contained 50 mM glucose and 0.3, 1.0, or 3.0 mM hydrogen peroxide, and was incubated for 1.5 h at 37°C using a shaker. The cells were removed by centrifugation to terminate the reaction. To determine the remaining hydrogen peroxide concentration after the reaction with living cells, we used the oxidative coupling reaction with 4-aminoantipyrene catalyzed by horseradish peroxidase (Sedewitz et al., 1984). As a co-substrate, 3,5-dichloro-2-hydroxybenzene sulfonic acid sodium salt was used. The assay mixture contained 400 μ l of 5.9 mM 4-aminoantipyrene, 400 μ l of 21.1 mM 3,5-dichloro-2-hydroxybenzene sulfonic acid sodium salt, 4 μ l of 400 unit/ml horseradish peroxidase, and 50 μ l of supernatant containing hydrogen peroxide. Each assay mixture was added for the colorimetric reaction, at 25°C. After 5 min, we conducted a spectrophotometric measurement at 546 nm.

To determine the remaining hydrogen peroxide concentration after the reaction with cell-free extract and enzymes, a reaction mixture containing 1 mM hydrogen peroxide and 6% (v/v) cell-free extracts was incubated at 37°C for 1 h. The remaining hydrogen peroxide in the reaction mixture was determined by ferrithiocyanate colorimetric assay (Thurman et al., 1972). 25 μ l of reaction was initiated by the addition of NADH-dependent or -independent activity terminated by adding 975 μ l of 5% trichloroacetic acid. After the removal of precipitated protein by centrifugation, 0.1 ml of 10 mM ferrous ammonium sulfate and 0.5 ml of 2.5 M potassium thiocyanate were added. We measured the absorption of the red ferrithiocyanate complex at 480 nm.

NADH- or NADPH-independent activity corresponding to catalase was also determined by monitoring the decrease in the absorption of hydrogen peroxide at 240 nm in 50 mM sodium phosphate buffer (pH 7.0) (Aebi, 1983) for enzymology.

NADH- or NADPH-dependent activity corresponding to NAD(P)H peroxidase was also determined by monitoring the decrease of the absorption of NADH or NADPH

at 340 nm in 50 mM sodium phosphate buffer (pH 7.0) (Dolin, 1953) for enzymology.

Preparation of cell-free extracts. For the detection of enzyme activities in a cell-free extract, approximately 3 g of the wet weight of the cells was re-suspended in 12 ml (4 times the value of wet cells) of 50 mM sodium phosphate buffer (pH 7.0) and then disrupted by two passages through French pressure cells (SLM-AMINCO; Spectronic Instruments) at 2,000 kg/cm².

Phenylmethanesulfonate (final concentration 2.0 mM) and 9 mg lysozyme (0.3% of wet cell weight) was added to the suspension before and after the passage through the high-pressure homogenizer, and sonicated for 4 min at 4°C. The resultant suspension was centrifuged at $6,000 \times g_{\max}$ (Avanti HP-25 centrifuge, Beckman) for 20 min at 4°C to remove unbroken cells. The cell-free extracts were dialyzed three times against 3 L of 50 mM sodium phosphate buffer.

For enzyme purification from cell-free extracts, approximately 30 g of the wet weight of the cells underwent the same methods before dialysis, and continued to the next purification step.

Enzyme purification. All purification steps were performed below 4°C. Ammonium sulfate (260 g/l) was added to the cell-free supernatant. After centrifugation at $64,000 \times g_{\max}$ (Avanti HP-25 centrifuge, Beckman) for 30 min, the supernatant was subjected to a Toyopearl Butyl-650S (Tosoh, Japan) column (CV: 500 ml) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 260 g/l ammonium sulfate. The bound protein was eluted with a liner gradient from 260 g/l to 0 g/l ammonium sulfate. The initial peak was an NADH-dependent active fraction and the subsequent peak was an NADH-independent active fraction. The two active fractions were separately pooled and dialyzed against 3 L of 50 mM sodium phosphate buffer (pH 7.0) three times.

Each dialysate was subjected separately to a DEAE-Sephacrose Fast Flow (GE Healthcare Bio-Sciences, USA) column (CV: 80 ml) pre-equilibrated with 50 mM sodium phosphate (pH 7.0) containing 3 μ M FAD. The unbound protein was eluted with 3 column volumes of the same buffer, and enzyme fractions were eluted with a linear gradient with 5 column volumes of NaCl (0–250 mM), 3 column volumes of 50 mM sodium phosphate (pH 7.0) containing 3 μ M FAD, and 250 mM NaCl, a linear gradient with 5 column volumes of NaCl (250–1,000 mM). Each active fraction from the NADH-dependent active fraction and the NADH-independent active fraction was pooled, and then dialyzed against 3 L of 50 mM sodium phosphate buffer (pH 7.0) three times.

After dialysis, each dialysate was subjected separately to a hydroxyapatite column (Wako Ltd., Japan, CV: 40 ml) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0) with 3 μ M FAD. The enzyme was eluted with a linear gradient with 3 column volumes of sodium phosphate buffer (50–100 mM), with 3 column volumes of the 100 mM pH 7.0 phosphate buffer containing 3 μ M FAD, with a linear gradient with 3 column volumes of sodium phosphate buffer (100–200 mM), with 3 column volumes of the 200 mM pH 7.0 phosphate buffer containing 3 μ M FAD, and with a linear gradient with 3 column volumes

of sodium phosphate buffer (200–300 mM). Each NADH-dependent and NADH-independent active fraction was separately pooled and concentrated. The purity and molecular weight of the enzymes were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As the need arose, each active fraction was subjected to a POROS HQ-H (Applied Biosystems, Japan) column (1.0 cm $\times$ 10.0 cm) pre-equilibrated with 50 mM sodium phosphate (pH 7.0), and eluted with a linear gradient of 60 column volumes of NaCl (0–1,000 mM). To determine the N-terminal amino acid sequences of proteins, the proteins were electro-transferred to a polyvinylidene difluoride membrane and the sequences were determined using a protein sequencer (PPSQ-31A, Shimadzu).

Genetic analysis. The degenerated polymerase chain reaction (PCR) primers (Fig. S3) were used for the first PCR, and *P. pentosaceus* Be1 genome DNA was extracted and used as the template DNA. The PCR fragments obtained were cloned into pT7 Blue vector (Novagen, Tokyo, Japan) and sequenced. Sequence analysis indicated that the 430 bp and 330 bp fragment encoded parts of the NADH peroxidase and the Mn-catalase of *P. pentosaceus* Be1, respectively. Their sequences showed a high homology to those of the presumed genes of NADH peroxidase and Mn-catalase from *P. pentosaceus* ATCC 25745, and we determined the full DNA sequences of the genes of two enzymes of *P. pentosaceus* Be1 by using the primers based on the genome sequences of *P. pentosaceus* ATCC 25745.

The 16S rRNA gene sequence of 1438 nucleotides was determined for the strain Be1 as described by Shida et al. (1997).

Nucleotide sequence accession numbers. The 16S rDNA sequence of *Pediococcus pentosaceus* Be1, and the DNA sequences of the genes of the two enzymes, NADH peroxidase (1,371 bp) and Mn-catalase (801 bp) of *P. pentosaceus* Be1, were submitted to the DNA Data Bank of Japan under accession numbers LC076286, AB786727 and AB786728, respectively.

Results and Discussion

Although the enrichment medium contained sources for ROS, 66 strains of the isolates (Table 1) grew well in the enrichment medium, and were isolated from various fermented foods following five cycles of plate culture in aerobic conditions using the enrichment medium (Table 1). Next, we measured the scavenging activity for 3 mM hydrogen peroxide in the 66 isolates and found 17 strains having activity. Finally, we succeeded in isolating one strain, strain Be1, from the Japanese traditional food of salted radish aged with koji rice, which displayed the highest scavenging activity for 3 mM hydrogen peroxide of the 17 strains (Table 1). The addition of glucose was effective for the hydrogen peroxide scavenging reaction by *P. pentosaceus* Be1.

Based on the taxonomical characterization (Uchimura and Okada, 1992b) and the 16S rDNA sequence of this strain, we identified it to be *P. pentosaceus*. *P. pentosaceus* Be1 strain showed the highest scavenging activity among the tested lactic acid bacteria involved in fermented food

Fig. 1. Relationship between scavenging activity and survival rate of lactic acid bacteria in the presence of hydrogen peroxide.

Lactic acid bacteria were treated with 0, 0.3, 1.0, or 3.0 mM hydrogen peroxide for 1.5 h at 37°C. After treatment, the number of living cells (line graphs) and hydrogen peroxide scavenging activities in culture media (bar graphs) were measured. The values are the means ($\pm$ SD, $n = 3$) for each strain. (A): *P. pentosaceus* Be1 strain, (B): *L. plantarum* NRIC 1067^T and *L. acidophilus* NRIC 1547^T, (C): *S. thermophilus* NRIC 0256^T and *P. pentosaceus* NRIC 0099^T.

Table 2. Hydrogen peroxide scavenging activity by living cells and in cell free extracts.

	H ₂ O ₂ scavenging activity		
	Living cells*	Cell-free extracts	
		NADH peroxidase**	Catalase**
	(mmol•mg ⁻¹ (dry cell weight)hour ⁻¹)	(mU/mg protein)	(mU/mg protein)
<i>P. pentosaceus</i> Be1	2.8 $\pm$ 0.4	5.3 $\pm$ 0.5	44.7 $\pm$ 1.7
<i>P. pentosaceus</i> ATCC 25745	0.9 $\pm$ 0.1	2.3 $\pm$ 0.2	12.0 $\pm$ 7.2

H₂O₂ concentrations of its scavenging activity in living cells, NADH peroxidase and catalase in cell-free extracts are 3 mM, 1 mM and 1 mM, respectively.

*The values are means ($\pm$ standard deviation [SD], $n = 3$) for each strain.

**The values are the the means of two measurements of the individual enzyme activities.

(Fig. S1). Based on the high or low scavenging activity and taxonomy indicated in Fig. S1, we selected four typical strains of lactic acid bacteria—*P. pentosaceus* NRIC0099^T, *L. acidophilus* NRIC1547^T, *S. thermophilus* NRIC0256^T, and *L. plantarum* NRIC1067^T—for comparing with *P. pentosaceus* Be1 strain. We examined the relationship of tolerance and scavenging activity to hydrogen peroxide in the *P. pentosaceus* Be1 and four tested lactic acid bacteria strains. With the exception of *P. pentosaceus* Be1, the strains could not scavenge a high concentration of hydrogen peroxide (3.0 mM) (Fig. 1). *P. pentosaceus* Be1 showed potent peroxide scavenging activity and consistently retained the number of living cells under the tested conditions (Fig. 1A). *L. acidophilus* NRIC 1547^T and *L. plantarum* NRIC 1067^T underwent a reduction in the number of living cells at a hydrogen peroxide concentration at which they could not scavenge by themselves (Fig. 1B). Although the peroxide scavenging activity of *P. pentosaceus* NRIC 0099^T and *S. thermophilus* NRIC 0256^T was low and markedly low, respectively, under all tested conditions, they showed no reduction in the number of living cells (Fig. 1C).

These results suggest that there are two types of hydrogen peroxide-resistant strains that retain the number of

living cells after exposure to hydrogen peroxide: one shows hydrogen peroxide scavenging activity and the other does not. The strains with a potent peroxide scavenging activity showed a higher resistance against hydrogen peroxide at the tested hydrogen peroxide concentrations. In the tested strains, *P. pentosaceus* Be1 exhibited a resistance against extracellular hydrogen peroxide and the ability to break it down. It displayed the highest environmental hydrogen peroxide scavenging activity over a wide-range of concentration of hydrogen peroxide (0.3, 1.0, and 3.0 mM) among the lactic acid bacteria tested.

Scavenging activity was detected in the cultured cells, but not in the culture supernatant of the media. The activity in the cells was denaturated by heat treatment. These observations suggested that *P. pentosaceus* Be1 had intracellular peroxide scavenging enzyme(s). In addition, we measured the hydrogen peroxide scavenging activity in dialyzed cell-free extracts from aerobically cultured *P. pentosaceus* Be1.

Table 2 shows the NADH-dependent hydrogen peroxide scavenging activity (5.3 mU/mg protein) and the NADH-independent hydrogen peroxide scavenging activity (44.7 mU/mg protein) of the cell-free extracts obtained from *P. pentosaceus* Be1 cells. The NADPH-dependent

(A) Butyl Toyopearl 650S column chromatograph of *P. pentosaceus* Be1

I. NADH peroxidase

II. Mn-catalase

(B) SDS PAGE of purified enzymes

Fig. 2. Purification of NADH peroxidase and Mn-catalase from *P. pentosaceus* Be1.

Two active fractions, peak I (NADH peroxidase activity) and peak II (catalase activity) in a Butyl Toyopearl 650S (Tosoh) column chromatograph, were separately purified by following the chromatographic steps in Section “Materials and Methods.” After purification, the purity of each protein was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE I, II). The arrow indicates the purified enzyme from *P. pentosaceus* Be1.

Table 3. Purification of NADH peroxidase and Mn-catalase from *P. pentosaceus* Be1.

Step	Total activity (U)	Total protein (mg)	Sp act (U mg ⁻¹)	Fold	Recovery (%)
I. NADH peroxidase					
Extraction from cells	11.4	882.00	0.013	1.00	100.0
Ammonium sulfate fractionation	12.1	643.00	0.019	1.44	100.0
Butyl-Toyopearl 650S chromatography	8.8	9.40	1.030	78.80	72.4
DEAE-sepharose chromatography	1.0	1.30	0.830	63.20	8.5
Hydroxyapatite chromatography	7.2	0.75	1.920	145.00	12.5
II Mn-catalase					
Extraction from cells	65.0	1427.60	0.046	1.00	100.0
Ammonium sulfate fractionation	57.4	1067.20	0.054	1.17	88.4
Butyl-Toyopearl 650S chromatography	88.6	88.40	1.000	21.74	136.4
DEAE-sepharose chromatography	18.8	2.96	6.340	137.83	28.9
Hydroxyapatite chromatography	4.7	0.17	27.410	595.87	7.2

activity of the extract was too low to be detected. The NADH-independent activity of the extract could be due to Mn-catalase, because the activity was not inhibited by 10 mM sodium azide (Uchimura and Okada, 1992c). Although the NADH-dependent activity of the extract may

be similar to the reported activities of AhpC (Prx)-participating enzymes or AhpC (Prx)-nonparticipating enzymes, no Prx-dependent activity (Niimura et al., 2000) was found in the cell-free extracts (data not shown), and this could be due to NADH peroxidase.

MDKVKVI I VGASHGGHESAIELLDKYDNVDVTIYEMGDFVSFMSCGMQLYLEGDVTDVNS 60
 ---MKVIVLGS SHGGYEAEELLNLHPDAEIQWYEKGDFISFLSCGMQLYLEGKVKD VNS 57

Be1 :
 V583 :

VRNFRPEDITSKGGKIYNNHQVTAIDVDQKQVTVKDLKAGSEEQVAYDKLILSSGVTPKN 120
 VRYMTGEKMESRGVNVSNTETAIQPKEHQVTVKDLVSGEERVENYDKLIISP GAVPFE 117

Be1 :
 V583 :

LPVP GTDLKNVYLMPRGYDWATKIKAAALTDDSIKNVAVIGSGYTIGIEAAEVFAKKGKHVTL 180
 LDIPGKDL DNIYLMRGRQWAIKLKQKTVDPEVNNVVVIGSGYTIGIEAAEF AKAGKKVTV 177

Be1 :
 V583 :

FD FID RPLGNYNHEMTEIIDKLTLDNGIQVEMSQSITAFKGDDKVASVETKRGSYPADL 240
 ID ILDRPLGVYLDKEFTDVLTEEMEANNITIATGETVERYEGDGRVQKIVTDKNAYDADL 237

Be1 :
 V583 :

VIAAAGVQPNTEWLKGVVDLTDRGFINVDGYLR TNVPDVFAIGDAAILPLSIPAGKPSPITA 300
 VVVAAGVRPNTAWLKGTLELHPNGLIKTDEYMRTSEP DVFAVGDA TL IKYNPADTEVNIA 297

Be1 :
 V583 :

LATTARREAGYVVNHI FEKKPTQIFKGVVGSALHV FDEHFASTGVNEFTAERSNVEIVN 360
 LAT NARKQGRFAVN KLEE---PVKPFPGVQGSSGLAVFDYKFAS TGINEVMAQKLKGETKA 355

Be1 :
 V583 :

SHYVDHIRPAYVPKAEGNVRVDVIDTFDPHTHIVLGGA VLSTADMTAQGNVLALAVEHRL 420
 VTVVEDYLMDFNPDKQ---KAWFKLVYPDETTQILGAQLMSKADLTANINAISLA IQAKM 412

Be1 :
 V583 :

TVEDLAEADFFFQPGYDRQWSLINLAAQH ALGYARF 456 Be1 : *Pediococcus pentosaceus* Be1
 TIEDLAYADFFFQPAFDKPWNINTAA LEAVKQER- 447 V583 : *Enterococcus faecalis* V583

 : Redox active site
 : fAD binding site
 : NAD binding site

A). NADH peroxidase amino acid sequence

(A). NADH peroxidase amino acid sequence

Be1	MFKHTRKRLQYNAKPNHPDPI	MARRLQEALGGQWG	ETTGMMSYLSQGWASTGDEKYKDLLL	60
ATCC14431	MFKHTRKRLQYNAKPDSDPI	MARRLQESLGGQWG	ETTGMMSYLSQGWASTGAEKYKDLLL	60
	*****:.:*****:*****:*****:*****			
Be1	DTGTEETIAFVEMISTMIGYLLEAPFSAEDLKRDP	SLAVTMAGMDPEHALVHGLNASLNN		120
ATCC14431	DTGTEETMAFVEMISTMIGYLLEDAPFGPEDLKRDP	SLATTMAGMDPEHSLVHGLNASLNN		120
	*****:*****:***.*****:*****:*****:*****			
Be1	PNGSAWNADYVTSSGNLVADMRFNVVRE	SEARLQVSRLYSMTEDEGIRDMLKFLARETQ		180
ATCC14431	PNGAAWNAGYVTSSGNLVADMRFNVVRE	SEARLQVSRLYSMTEDEGVRDMLKFLARETQ		180
	:*.*****:*****:*****:*****:*****			
Be1	HQLQFMKAQEELEEKYGVIVPGDMKDI	EHSEFSHVL MNFSDGDGSKAFEGQIARDGEKFT		240
ATCC14431	HQLQFMKAQEELEEKYGIIVPGDMKEI	EHSEFSHVL MNFSDGDGSKAFEGQVAKDGEKFT		240
	*****:*****:*****:*****:*****:*****			
Be1	YQENPEAMSSIPHIKPADPKLHNDQG			266
ATCC14431	YQENPEAMGGIPHIKPGDPRLHNHQG			266
	*****.*****.**:***.**			

Bel : *Pediococcus pentosaceus* Bel
ATCC14431 : *Lactobacillus plantarum* ATCC14431

 : Manganese binding site

(B). Mn-catalase amino acid sequence

Fig. 3. Deduced amino acid sequence of NADH peroxidase and Mn-catalase from *P. pentosaceus* Be1.

The deduced amino acid sequences of each enzyme from *P. pentosaceus* Be1 (top line) were compared with that of each corresponding enzyme (bottom line). Identical residues are asterisked; similar amino acid residues are indicated by dots. Each binding site was referred to the UniProt website data of Mn-catalase (P60355) and NADH peroxidase (P37062). The sites are boxed in the figure. (A): deduced amino acid sequence of NADH peroxidase from *P. pentosaceus* Be1 and *Enterococcus faecalis* V583, (B): deduced amino acid sequence of Mn-catalase from *P. pentosaceus* Be1 and *L. plantarum* ATCC14431.

Based on the NADH-dependent and -independent activity for the hydrogen peroxide scavenging reaction, we successfully purified the two types of hydrogen peroxide scavenging enzymes to homogeneity in SDS-PAGE from the extracts (Fig. 2, Table 3). Enzymatic characterization of the two enzymes is as follows. NADH-dependent hydrogen peroxide scavenging enzyme: The enzyme exhibited no activity without NADH acting as the electric donor, and shows a specific absorption spectrum typical of a flavoprotein (Fig. S2). Hydrogen peroxide is a specific substrate and not active for oxygen, *t*-butyl hydroperoxide, and cumene hydroperoxide. Its optimum pH was between 4.0 and 5.5 at 20 to 40°C. NADH-independent hydrogen peroxide scavenging enzyme: This enzyme does not need an electric donor for reaction, and shows no specific absorption spectrum typical of a flavoprotein or heme-protein (Fig. S2). The addition of an inhibitor of heme-catalase, NaN₃ (1 mM) and NaCN (1 mM) had no effect on enzyme activity. Hydrogen peroxide is a specific substrate and is not active for oxygen, *t*-butyl hydroperoxide, and cumene hydroperoxide. Its optimum pH was between 4.5 and 9.0 at 30 to 50°C.

These enzymatic characterizations of the two enzymes indicated that NADH-dependent and NADH-independent hydrogen peroxide scavenging enzymes belonged to NADH peroxidase and Mn-catalase, respectively. To confirm the nomenclature of the two enzymes, their genes were analyzed.

The *N*-terminal amino acid sequences of *P. pentosaceus* Be1's NADH-dependent and -independent hydrogen peroxide scavenging enzyme were determined by a protein sequencer (Fig. S3). We found that the *N*-terminal amino acid sequences of NADH peroxidase and catalase (PEPE_1619 and PEPE_0969, respectively) deduced from the genome sequence of *P. pentosaceus* ATCC 25745 are highly homologous to those of *P. pentosaceus* Be1's presumed NADH peroxidase and presumed Mn-catalase (Fig. S3). Based on the determined *N*-terminal amino acid sequence and the genomic information of *P. pentosaceus* ATCC 25745, we designed PCR primers as indicated in Fig. S3, and cloned the corresponding genes of *P. pentosaceus* Be1.

Finally, we sequenced the full DNA of the genes of the two enzymes: the presumed NADH peroxidase gene (1,371 bp) and the presumed Mn-catalase gene (801 bp) of *P. pentosaceus* Be1 (Fig. 3). Their deduced amino acid sequences were 100% identical to those deduced for NADH peroxidase and Mn-catalase of *P. pentosaceus* ATCC 25745, respectively (data not shown). The enzymes of *P. pentosaceus* ATCC 25745 have not yet been purified and characterized. We then compared them with the already characterized enzymes. The presumed NADH peroxidase's sequence showed a 46% similarity to that of *E. faecalis* V583 (Ross and Claiborne, 1991), and their redox active, FAD binding, and NAD binding sites were completely consistent (Fig. 3A). The presumed Mn-catalase's sequences showed a 91% similarity to that of *L. plantarum* ATCC 14431 (Igarashi et al., 1996), and their manganese binding sites were completely consistent (Fig. 3B). These similarity and enzymology data strongly indicate that the purified NADH-dependent and NADH-independent hydro-

gen peroxide scavenging enzymes in this study belonged to NADH peroxidase and Mn-catalase, respectively.

Mn-catalase has been purified from several microorganisms (Allgood and Perry, 1986; Amo et al., 2002; Barynin and Grebenko, 1986; Kagawa et al., 1999; Kono and Fridovich, 1983). On the other hand, although NADH peroxidase activity was first observed in *Enterococcus faecalis* (Dolin, 1953), the enzyme had not yet been purified from the original strain, and was purified from *Escherichia coli* when its gene was cloned and expressed in *E. coli* (Ross and Claiborne, 1991). The NADH peroxidase was first purified from the original strain in the work reported here. Furthermore, both of the hydrogen peroxide scavenging enzymes—NADH peroxidase and Mn-catalase—were first purified from the same original strain as described in this report. The cell-free extract obtained from *P. pentosaceus* Be1 showed a twofold NADH peroxidase activity and a threefold NADH catalase activity compared with that obtained from *P. pentosaceus* ATCC 25745 in the presence of 1 mM hydrogen peroxide (Table 2). The environmental hydrogen peroxide scavenging activity of *P. pentosaceus* Be1 was three times higher than that of *P. pentosaceus* ATCC 25745. The observed high scavenging activity against the environmental hydrogen peroxide of *P. pentosaceus* Be1 is thought to be due to the high expression of the two hydrogen-peroxide-degrading enzymes, both of which were purified in this study. Since gastrointestinal inflammation causes the generation of peroxide (Miyamoto et al., 2001), the scavenging of environmental hydrogen peroxide by lactic acid bacteria is expected to protect the tissues from damage caused by the peroxide. Because *P. pentosaceus* Be1 strain showed the highest scavenging activity for environmental hydrogen peroxide among the isolates in this study, and the tested lactic acid bacteria was involved in fermented food (Fig. S1), we believe that *P. pentosaceus* Be1 has a unique potential as a probiotic to prevent hydrogen peroxide generation.

Acknowledgments

We thank Y. Tamura, T. Fukaya, and Y. Watanabe of the Department of Bio-Science, Tokyo University of Agriculture for their technical assistance. This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary Materials

Fig. S1. Scavenging activity of lactic acid bacteria.

Lactic acid bacteria were treated with 0, 0.3, 1.0, or 3.0 mM hydrogen peroxide for 1.5 h at 37°C. After treatment, hydrogen peroxide concentrations in the culture media were measured as described in Section "Materials and Methods." The values are the means (±SD, *n* = 3) for each strain.

Fig. S2. Absorption spectrum of NADH peroxidase and Mn-catalase from *P. pentosaceus* Be1.

Absorption spectrum of the purified NADH peroxidase and Mn-catalase in 50 mM sodium phosphate buffer (pH 7.0), at 25°C. (A): absorption spectrum of NADH peroxidase from *P. pentosaceus* Be1, (B): absorption spectrum of Mn-catalase from *P. pentosaceus* Be1.

Fig. S3. *N*-terminal amino acid sequences of purified enzymes from *P. pentosaceus* Be1, and polymerase chain reaction (PCR) primers designed from the *N*-terminal amino acid sequences of *P. pentosaceus* Be1 and genomic information of *P. pentosaceus* ATCC 25745.

The chemically determined amino acid sequences of each enzyme from *P. pentosaceus* Be1 (top line) were compared with the deduced amino acid sequences of each corresponding enzyme from *P. pentosaceus* ATCC 25745 (bottom line). Identical residues are asterisked. The amino acid sequence used in PCR primers is shown by the solid underline. (A): NADH peroxidase, and (B): Mn-catalase.

Supplementary figures are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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