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Identification of Antioxidant(s) from Lactic Acid Bacteria and Screening of High Antioxidative Strain(s)

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Research aims

Oxygen is a powerful oxidant and therefore a good electron acceptor for respiration metabolism. However oxygen can also be toxic to all living organisms. Incomplete reduction of oxygen generates reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals, which can have deleterious effects on biomolecules including DNA, RNA, membrane lipid, and protein. Although higher organisms including human have developed their own defense systems against ROS, many clinical and epidemiological studies have suggested that intake of dietary antioxidants, including polyphenolic compounds, Vitamins E and C, and carotenoids, have an important role in the prevention of the oxidative stress related diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders.[1)]

Lactic acid bacteria (LAB) are a group of bacteria which convert more than fifty percents of carbohydrate to lactic acid through fermentation metabolism, and have been studied for their possible beneficial physiological effects on human and animals. To date, various health promoting effects of LAB such as intestinal regulator activity, immune-potentiating activity, anti-tumor activity, and resistance to pathogens, have been reported. Recent several studies have shown that ingestion of LAB alleviates pathological condition of oxidative stress related model diseases, implying that LAB can serve as antioxidant to animals.[2,3)] To date, several antioxidant enzymes, such as manganese superoxide dismutase and NADH peroxidase, have been reported in LAB. We previously found a ferritin-like iron-binding protein (designed as Dpr) as a new LAB antioxidative protein, and demonstrated that Dpr confers oxygen tolerance to LAB by sequestering intracellular free iron ions which otherwise promotes generation of hydroxyl radicals through fenton reaction.[4)] During the course of study on Dpr, we also detected activity, which inhibits fenton reaction, in low molecular weight fraction of cell-free extract. In case of bacteria including LAB, few low molecular antioxidants except glutathione have been reported, though many proteinous antioxidants have been extensively studied. Since low molecular weight antioxidants derived from LAB is expected to be highly useful for food industry and human health, it is important to verify the chemical nature of the antioxidant(s). To identify low molecular weight antioxidants from LAB, we screened high antioxidative LAB strains from our laboratory stock and analyzed properties of antixoidative activities of the selected strains.

Methods

LAB cell-free extracts were prepared as follows. LAB strains were grown in M17 or MRS broth at 30 or 37 degrees. An overnight culture of each LAB strain was diluted 100-fold by fresh medium, and then incubated for 24 hrs. LAB cells were collected by centrifugation at 8,000 g for 10 min and washed with 50 mM potassium phosphate buffer pH 7.0 (for ORAC assay) or 50 mM MOPS-NaOH buffer pH 7.0 (for TBARS assay) two times. The LAB cell pellets were then suspended in either 50 mM potassium phosphate buffer pH 7.0 (for ORAC assay) or 50 mM MOPS-NaOH buffer pH 7.0 (for TBARS assay), and the LAB cells was disrupted by the BeadBeater at 4,200 rpm for 2 min. The cell debris and Zirconium beads were removed by centrifugation at 13,000 g for 15 min. The supernatant was used as cell-free extract sample for TBARS and ORAC assays and the protein concentration of each extract was determined by BCA assay.

TBARS assay was performed as follows. The basal reaction mixture contained 10 mM MOPS-NaOH buffer (pH 7.0), 63 mM NaCl, and 4 mM 2-deoxyribose in a total volume of 0.3 ml. To the basal reaction mixture was added cell free extract of LAB to give a final concentration of 10 μg/ml. The reaction was started by adding hydrogen peroxide and ferrous ammonium sulfate to the reaction mixture at final concentrations of 100 and 10 μM, respectively. After incubation at 37°C for 15 min, 0.25 ml of 1% (wt/vol) thiobarbituric acid and 2.8% (wt/vol) trichloroacetic acid were added to the reaction mixture. After the mixture was boiled for 10 min and rapidly cooled, the amount of chromogen formed in the sample was measured by its fluorescence emission at 553 nm (ex 532 nm).

ORAC assay was performed by the method described by Gillespie *et al*.[3)] ORAC value of cell free extract of LAB was calculated by using trolox, a standard antioxidative compound, as a control, and is indicated as μmol trolox equivalent (TE) per gram total protein.

Results

Antioxidative activity of cell-free extract of LAB was measured by TBARS and ORAC assays as described in methods. TBARS assay was used to detect both hydroxyl radical scavenging activity and inhibitory effect on iron-de-

Table 1. Antioxidative activity of intracellular cell-free extract of lactic acid bacteria screened by TBARS assay

Strain	Inhibition (%)
Lactobacillus sakei PP6-S	69.5
Lactobacillus casei JCM8129	64.7
Leuconostoc mesenteroides PP9-1	61.3
Pediococcus pentosaceus JCM5890	60.7
Lactobacillus paraplantarum PP7-4	60.2
Leuconostoc mesenteroides HS86-1	59.1
Lactobacillus sakei PP2-2	56.3
Lactobacillus casei JCM1134	55.2
Lactobacillus plantarum PP7-3	53.6
Leuconostoc lactis HS32-1	51.4

A list of 10 high antioxidative activity strains, out of 100 strains tested, is shown.

Table 2. Antioxidative activity of intracellular cell-free extract of lactic acid bacteria screened by ORAC assay

Strain	μmol TE/g total protein
Lactobacillus casei JCM20024	572
Lactobacillus sakei PP4-S	566
Lactobacillus plantarum HS47-1	469
Lactobacillus plantarum JCM1149	466
Lactobacillus rhamnosus ATCC53103	457
Lactobacillus plantarum HS66-1	422
Lactobacillus paracasei JCM2769	412
Leuconostoc mesenteroides PP9-1	394
Lactobacillus casei JCM1134	394
Lactobacillus sakei PP4-1	392

A list of 10 high antioxidative activity strains, out of 72 strains tested, is shown.

pendent hydroxyl radical formation (Fenton reaction). ORAC assay is a method of measuring antioxidative capacity of biological sample, and is used to measure antioxidative capacity of foods in the United States Department of Agriculture (USDA).[5,6] Laboratory stock of LAB strains including 41 strains of *Lactobacillus*, 9 strains of *Streptococcus*, 20 strains of *Enterococcus*, 14 strains of *Lactococcus*, 13 strains of *Leuconostoc*, 3 strains of *Pediococcus*, were tested for their antioxidative activities. All 100 strains tested by TBARS assay demonstrated antioxidative activity with inhibition rate in the range from 9.5 to 69.5%. The average inhibition rate was 32.8±12.9%. Among 100 strains, 10 high antioxidative strains were shown in Table 1. *Lactobacillus sakei* PP6-S which isolated from a fermented fish food product showed highest activity out of 100 strains.

ORAC assay was performed to measure total antioxidant capacity of cell-free extract of LAB. All 72 strains tested by ORAC assay demonstrated detectable ORAC values in the range from 82.5 to 572.2 μmol TE/g total protein. The average ORAC value of 72 strains was 258.4±107.6 TE/g total protein. Table 2 shows a list of 10 strains which demonstrated high ORAC values. *Lactobacillus casei* JCM20024 showed highest ORAC value out of 72 strains tested. Among the 10 strains listed in Table 2, all the strains were *Lactobacillus* except *Leuconostoc mesenteroides* PP9-1.

We next analyzed properties of antixodative activities of cell free extract of these high antioxidative strains. As the result of fractionation of cell-free extract by ultrafiltration, both antioxidative activities measured by TBARS and ORAC assays were recovered in low molecular weight fraction (less than 10 KDa) with the range of 32–77%. Antioxidative activity detected by TBARS was resistant to heat treatment (boiling for 10 min) and Protenase K treatment. ORAC value of cell-free extract was also relatively heat stable. Interestingly, Protenase K treatment increased ORAC values of the cell-free extract by about 2.8-fold compared to that of the non-treated sample, suggesting that peptide derived from LAB protein might have antioxidative activity.

Conclusion

In this study we successfully detected anti-oxidative activity from cell-free extract of 100 strains of LAB by using TBARS and ORAC assays. The highest antioxidative activity strain screened by TBARS assay was *Lactobacillus sakei* PP6-S. The strain which demonstrated highest ORAC value was *Lactobacillus casei* JCM20024. Several preliminary analyses indicated that antioxidative activities detected by TBARS and ORAC assays were low molecular weight compound(s) and relatively heat stable. We are currently trying to identify the chemical nature of the compound(s) of antioxidative activity from the strains screened by this study.

References

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