*Regular Article*

# Characterization of Exopolysaccharides Produced by Thermophilic Lactic Acid Bacteria Isolated from Tropical Fruits of Thailand

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**In the present study, we have obtained two exopolysaccharide (EPS)-producing thermophilic lactic acid bacteria (LAB) that were isolated from tropical fruits of Thailand. The two strains, designated LY45 and PY45, were identified as *Pediococcus pentosaceus* and *Lactobacillus amylovorus*, respectively. Both plant-derived LAB strains, which produce neutral EPSs together with the acidic one, can grow vigorously at 45°C and even at 50°C. Hyaluronidase (EC 3.2.1.35), which catalyzes the degradation of hyaluronic acid, activates an inflammatory reaction. Interestingly, EPSs produced by the LY45 and PY45 strains were found to inhibit hyaluronidase activity at the same order of $IC_{50}$ values as did sodium cromoglicate and dipotassium glycyrrhizinate, which are well-known as anti-inflammatory agents. The LY45-derived neutral EPS consists of glucose and mannose as monosaccharide components, whereas the acidic one contains mainly mannose, together with glucose and galactose. On the other hand, although *Lactobacillus amylovorus* PY45 also produces neutral and acidic EPSs, the main monosaccharide in both EPSs is mannose, and glucose is a minor component. Furthermore, the PY45 strain may be probiotically and industrially useful because the microorganism can utilize starch and glycogen as carbon sources.**

**Key words** exopolysaccharide; *Lactobacillus amylovorus*; *Pediococcus pentosaceus*; thermophilic lactic acid bacteria; anti-inflammatory substance

Traditionally, lactic acid bacteria (LAB) have been useful for producing fermented foods, such as yogurt, kimchi, and cheese. LAB—Gram-positive bacteria—are non-pathogenic and are generally recognized as safe (GRAS) microorganisms; therefore, they have been the focus of attention for their industrial importance.[1)]

Microorganisms that contribute to human health are called *probiotics*. This word is internationally defined as "living microorganisms conferring a health benefit on the host when administered in adequate amounts."[2)] We have isolated many kinds of LAB strains from plant sources such as vegetables, fruits, flowers, and medicinal plants to establish a plant-derived LAB library with more than 600 strains. We have demonstrated that some strains stocked in the LAB library are useful for enhancing intestinal immunity, improving liver function, and preventing metabolic syndrome.[3–6)]

LAB fermentation often proceeds even at low temperatures, causing the overgrowth of LABs. As the result, the degradation of flavors is proceeded and affected with taste of the fermented foods. Since thermophilic LAB strains hard to ferment at lower temperatures, therefore, if the thermophile ones are successfully isolated, they will contribute to prevent over-fermentation. In the present study, we isolated 28 strains of thermophilic LAB from tropical fruits of Thailand. In the isolates, we found two exopolysaccharide (EPS)-producing LAB strains. The LY45 strain was isolated from lychee, and the PY45 strain was obtained from pineapple. These strains were identified as *Pediococcus* (*P.*) *pentosaceus* and *Lactobacillus* (*Lb.*) *amylovorus*, respectively.

In general, *P. pentosaceus* can grow at temperatures up to 40°C but not to 50°C.[7,8)] However, the optimal temperature for the growth of *P. pentosaceus* LY45 is 45°C, and it can grow at temperatures up to 50°C. On the other hand, EPS-producing *Lb. amylovorus* PY45 can utilize starch as a carbon source.

Some strains of LAB contribute to human health through the production of bioactive compounds such as EPSs.[9,10)] EPSs have been shown to benefit human health by their immunomodulation, anti-gastritis, anti-ulcer, and anti-virus activities.[11–13)] We have shown that *P. pentosaceus* LP28 has the strain-specific EPS biosynthetic gene cluster,[14)] and intake of the strain is effective to improve the high fat diet-induced obesity and fatty liver of the mice.[6)] Furthermore, the strain is also useful to reduce body fat and body weight in human clinical study.[3)]

In the present study, EPSs produced by the both LY45 and PY45 strains were shown to inhibit the activity of hyaluronidase, which contributes to early inflammatory reactions. In addition, we chemically characterized the EPSs produced by two thermophilic LAB strains.

## MATERIALS AND METHODS

**Media and Growth Conditions** MRS broth (Merck KGaA, Darmstadt, Germany) was the medium used to grow all LAB strains. A semi-defined medium (SDM)[15)] without a yeast nitrogen base but supplemented with a 0.2% (v/v) vitamin solution and a 0.1% (v/v) trace element solution[16)] was called a modified SDM. This medium was used to evaluate

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the EPS productivity of LAB strains. To monitor cell growth, the pH and cell numbers (colony-forming unit (CFU)/mL) of the culture broth were measured at 3-h intervals.

**Isolation of Thermophilic LAB from Tropical Fruits and the Identification of EPS-Producing Strains** To isolate LAB, pieces of tropical fruits from Thailand were suspended in MRS broth and incubated anaerobically at 30, 37, or 45°C for 3 d. An aliquot of each culture broth was spread onto the MRS agar plate and incubated at a given temperature until a colony was formed. Each colony formed on the plate was re-spread onto a fresh MRS agar plate to purify the colony.

Thermophilic LAB strains, which can normally grow at 45°C, were obtained as follows: LAB candidates isolated as described above were re-cultured in the MRS broth at 45°C for 18 h. After cultivation, a 100-$\mu$L aliquot of the culture broth was re-inoculated into fresh MRS broth to grow at 45°C for 3 d. The number of CFU was monitored at 24-h intervals. Strains that display $10^8$–$10^9$ CFU/mL in the culture broth after 2 d of cultivation were nominated as thermophilic LAB.

To taxonomically identify the isolated strains, a partial nucleotide sequence of a 16S ribosomal DNA (rDNA) gene fragment, which was PCR-amplified using primers F27 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and R1492 (5′-GGC TAC CTT GTT ACG ACT T-3′), was determined as described previously[17–19] and then compared with the bacterial 16S rDNA database of the DNA Data Bank of Japan (DDBJ) by using the BLAST algorithm[20] and utilizing the non-redundant database provided by the National Center for Biotechnology Information (NCBI).

**DNA Preparation and Manipulation** The chromosomal DNA from the LAB strains was isolated using a CloneSaver Card (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). This, briefly, was the method: 10 $\mu$L of cell suspension cultured for 16 h was loaded onto a CloneSaver Card and allowed to dry at room temperature. A loaded sample disk was removed from the card by punching a disk out of the loaded sample area, placed in a PCR tube, and washed twice with 200 $\mu$L of FTA Purification Reagent (GE Healthcare). The disk was further washed twice with a TE buffer (10 mM Tris–HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0) and dried at 55°C for 10 min before the amplification of 16S rDNA.

**Culture Conditions for EPS Production by the LAB Strains** LY45 and PY45 strains produce EPSs. For the seed culture, a portion of each frozen stock culture was inoculated into an MRS medium and grown at 45°C for 48 h in the standing culture. The seed culture broth was inoculated at 0.2% (v/v) into the modified SDM containing the given concentrations of sugar and incubated at 45°C for 1–5 d in the standing culture.

**Purification of EPSs from LAB Culture Broth** The purification of EPSs is performed as follows: 81 mL of 100% (w/v) trichloroacetic acid (TCA) was added to 2 L of culture broth. After mixing at 4°C for 30 min, the cell mass and its debris were removed by centrifugation at 12000×$g$ for 10 min at 4°C, added to 2 L of acetone, and incubated overnight at 4°C. After centrifugation at 12000×$g$ for 10 min at 4°C, the precipitated crude EPS was washed with 100 mL of 70% (v/v) ethanol, followed by drying. The resulting pellet was dissolved in 30 mL of 50 mM Tris–HCl buffer (pH 8.0) with agitation for 1 h at 4°C, and debris was removed by centrifugation at 20000×$g$ for 30 min at 4°C. After adding to the supernatant fluid each 300-$\mu$L aliquot containing 1 mg of deoxyribonuclease I (Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.) and ribonuclease A (Nacalai Tesque, Kyoto, Japan) in 1 mL of the same buffer, the mixture was incubated at 37°C for 8 h. The incubation mixture was added with 300 $\mu$L of 2 mg/mL proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) contained in the same buffer to the mixture and followed by further incubation at 37°C for 16 h. After keeping the incubated mixture on ice, 81 mL of 100% (w/v) TCA was added and kept on ice for 1 h. The protein and debris were removed by centrifugation at 20000×$g$ for 30 min at 4°C, and the resulting supernatant fluid was mixed with 105 mL of 100% (v/v) ethanol. After centrifugation at 15000×$g$ for 5 min at 4°C, the precipitant was washed with 20 mL of 70% (v/v) ethanol and dried. The pellet was completely dissolved into 10 mL of distilled water, and the resulting EPS solution was dialyzed against the distilled water using a dialysis membrane (MWCO=10 kDa). The EPS concentration was determined by using the phenol sulfate method,[21] and the dialysate was lyophilized to obtain purified EPSs.

The EPSs obtained were further purified as follows: the lyophilized EPSs were dissolved in a 50 mM Tris–HCl buffer (pH 8.0) and applied to a TOYOPEARL DEAE-650M column (2.5×22 cm; Tosoh Bioscience, Japan) equilibrated with the same buffer. The column work was carried out at a flow rate of 1 mL/min at room temperature. The EPSs were eluted with a linear gradient of NaCl: 0 to 240 min, 0 mM; 240 to 600 min, 0–500 mM in the same buffer. The EPSs in each fraction (6 min/tube) were detected by the phenol sulfate method, and EPS-containing fractions were pooled, dialyzed against the distilled water (MWCO=10 kDa), and lyophilized to obtain purified neutral EPSs and acidic EPSs.

**Hyaluronidase Activity Inhibitory Assay** The hyaluronidase activity inhibitory assay was performed using the method of Fujitani *et al.*[22] with slight modifications: after the EPSs or some inhibitors were dissolved in 10 $\mu$L of water at the given concentration, a 5-$\mu$L portion of hyaluronidase solution (MP Biomedicals, Santa Ana, CA, U.S.A.) that contained 4 mg of the enzyme in 1 mL of 100 mM sodium acetate buffer (pH 4.0) was added. After incubation of the mixture at 37°C for 20 min, a 10-$\mu$L portion of 0.5 mg/mL Compound 48/80 solution (MP Biomedicals) that contained 3.75 mg $CaCl_2 \cdot 2H_2O$ in 1 mL of the same buffer was added to the mixture and followed by the further incubation at 37°C for 20 min. A 25-$\mu$L portion of hyaluronic acid solution (Wako Pure Chemical Industries, Ltd.) that contained 0.8 mg in 1 mL of the same buffer was added and kept at 37°C for 40 min. To stop the reaction, after 10 $\mu$L of 400 mM NaOH was added to the mixture, the sample was further combined with 10 $\mu$L of 100 mM potassium borate buffer (pH 10.0), heated at 100°C for 3 min, and then immediately placed in an ice bath. A *p*-dimethylaminobenzaldehyde solution (*p*-DMAB, Wako Pure Chemical Industries, Ltd.) was prepared by diluting the 10× stock solution (5 g of *p*-DMAB, 6 mL of 10 M HCl, and 44 mL of acetate) with acetate. The *p*-DMAB solution was mixed with each reacted sample in a 4 : 1 ratio, and the absorbance at 585 nm of each sample was measured using a VersaMax Microplate Reader (Molecular Devices, Silicon Valley, CA, U.S.A.). As a control, hyaluronidase-lacking reaction samples were used. The inhibitory percentage of hyaluronidase activity was calculated from the following formula:

Table 1. Numbers of LAB Candidates Isolated from Tropical Fruits in Thailand

| Source (scientific name) | Province | Numbers of candidates at different temperature | | |
|---|---|---|---|---|
| | | 30°C | 37°C | 45°C |
| Rambutan (*Nephelium lappaceum* L.) | Nakhon Pathom | 3 | 4 | 2 |
| Lychee (*Litchi chinensis* Sonn.) | Nakhon Pathom | 6 | 2 | 6 |
| | Nakhon Phanom | 0 | 0 | 1 |
| Longkang (*Lansium domesticum* Corr.) | Nakhon Pathom | 4 | 3 | 3 |
| Jew's plum (*Spondian pinnata* L.f.) | Nakhon Pathom | 1 | 2 | 1 |
| | Uttaradit | 2 | 2 | 3 |
| | Sukhothai | 2 | 0 | 1 |
| Sugar palm (*Bolassus flabellifer* L.) | Bangkok | 3 | 3 | 4 |
| Papaya (*Carica papaya* L.) | Nakhon Pathom | 2 | 1 | 1 |
| | Uttaradit | 0 | 2 | 3 |
| Satol (*Sandoricum koetjape*) | Uttaradit | 2 | 3 | 9 |
| Pineapple (*Ananas commosus*) | Nakhon Pathom | 3 | 3 | 2 |
| | Uttaradit | 2 | 3 | 3 |
| | Bangkok | 3 | 3 | 5 |
| Sugar apple (*Annona squamosa* L.) | Bangkok | 2 | 1 | 2 |
| | Phrae | 4 | 4 | 6 |
| Dragon fruit (*Hylocerreus undatus* (Haw) Britt & Rose) | Nakhon Pathom | 3 | 2 | 1 |
| Salak (*Salacca zalacca*) | Chanthaburi | 1 | 1 | 5 |
| Mak mouv (*Atidesma velutinosum* Bl.) | Phitsanulok | 2 | 2 | 0 |
| Nom maew (*Rauwenhoffia siamensi* Scheff.) | Kalasin | 4 | 4 | 0 |
| Emblic mylablan (*Phyllanthus emblicca* L.) | Kalasin | 0 | 0 | 0 |
| | Phitsanulok | 2 | 0 | 4 |
| Kiffir lime (*Citrus hystrix*) | Uttaradit | 3 | 4 | 0 |
| Star fruit (*Averrhora carambola* L.) | Uttaradit | 6 | 3 | 3 |
| | Si Saket | 2 | 1 | 2 |
| Durian (*Durio zibethinus* Murray) | Uttaradit | 4 | 3 | 3 |
| | Rayong | 0 | 0 | 3 |
| | Si Saket | 0 | 0 | 2 |
| Mango (*Manaifera indica* Linn.) | Pathum Thani | 3 | 5 | 1 |
| | Uttaradit | 2 | 2 | 2 |
| | Suphanburi | 1 | 0 | 2 |
| Jack fruit (*Artocarpus herophyllus*) | Phrae | 3 | 4 | 2 |
| Banana (*Musa sapientium*) | Samut Sakhon | 1 | 4 | 3 |
| | Uttaradit | 2 | 2 | 2 |
| | Suphanburi | 1 | 0 | 1 |
| Guava (*Psidium guajava* L.) | Phrae | 3 | 2 | 2 |
| Spodilla (*Manikaro zapota* L.) | Phitsanulok | 4 | 0 | 0 |
| Pomelo (*Citrus maxima*) | Phitsanulok | 2 | 1 | 0 |
| Watermelon (*Citrullus lanatus*) | Phitsanulok | 1 | 1 | 5 |
| Muskmelon (*Manikaro zapota* L.) | Phitsanulok | 1 | 0 | 0 |
| Longan (*Dimocarpus longan*) | Nakhon Pathom | 0 | 1 | 1 |
| | Chiang Mai | 0 | 0 | 2 |
| Pomegranate (*Punica aranatum* L.) | Nakhon Pathom | 0 | 3 | 1 |
| Madagascar plum (*Flucourtia indica* (Bum.f.) Merr) | Phitsanulok | 2 | 0 | 1 |
| Jujube (*Zizyiphus mauritiana* Lamk) | Nakhon Pathom | 1 | 6 | 2 |
| | Kanchanaburi | 0 | 1 | 1 |
| | Bangkok | 1 | 2 | 2 |
| Lantern tree (*Baccaurea ramiflora*) | Chanthaburi | 0 | 0 | 3 |
| Jambolan plum (*Eugenia cumini* Duce) | Bangkok | 0 | 2 | 0 |
| Marium plum (*Bouea macrophylla* Griff) | Kalasin | 0 | 2 | 0 |
| | Nonthaburi | 0 | 0 | 0 |
| Tamarind (*Tamarindos indica* L.) | Srisakade | 0 | 3 | 0 |
| Madras thorn (*Pithecellobium dulce* (Roxb). Benth) | Bangkok | 2 | 2 | 2 |
| | Nakhon Ratchasima | 1 | 2 | 3 |
| | Suphanburi | 1 | 0 | 2 |
| Rose apple (*Eugenia javanica*) | Uttaradit | 1 | 1 | 0 |
| | Srisakade | 1 | 3 | 1 |
| | Bangkok | 0 | 0 | 0 |
| | Ratchaburi | 0 | 0 | 3 |
| Star gooseberry (*Phyllanthus acidus*) | Uttaradit | 0 | 2 | 0 |
| Mongosteen (*Garcinia mangostana* Linn.) | Uttaradit | 0 | 0 | 0 |
| | Nakhon Pathom | 0 | 0 | 0 |
| | Bangkok | 0 | 0 | 0 |
| | Chumphon | 0 | 0 | 1 |
| Total | | 100 | 107 | 120 |

$$\text{Inhibition (\%)} = 100 - (S/C) \times 100$$

where $C$ (control) means hyaluronidase activity without samples or inhibitors, and $S$ (sample) means hyaluronidase activity in the presence of samples or inhibitors.

To calculate the $IC_{50}$ value—the concentration causing a 50% inhibition of each compound—a dose-dependent curve was drawn by plotting the sample or inhibitor concentration ($X$ in the following equation) *versus* the percentage of inhibition ratio ($Y$ in the following equation). The value was calculated from the sigmoidal curve using the following logistical curve equation:

$$Y = \alpha / (1 + \beta e^{-\gamma X})$$

where $\alpha$, $\beta$, and $\gamma$ are given constants.

Dipotassium glycyrrhizinate, ketotifen fumarate, and sodium cromoglicate (Wako Pure Chemical Industries, Ltd.), which are known as anti-inflammatory reagents, were used as the controls for the inhibition assay. The fucoidan derived from *Laminaria japonica*, (Carbosynth Limited, Compton, Berkshire, U.K.), which is an anti-inflammatory polysaccharide, was also used as a control of the hyaluronidase inhibitory assay.

**Analysis of Constitution Sugars in EPSs** The compositions of monosaccharide in EPSs were analyzed with the alditol acetate derivatization method, using GC-MS.[23] After the purified EPS (5 mg) was hydrolyzed with 2 M trifluoroacetic acid at 120°C for 1 h to obtain each monosaccharide, the reduced reaction was performed, followed by acetylation. The resulting derivative was analyzed by GC-MS, using a JMS-T100GCV AccuTOF GCv 4G gas chromatograph high-resolution time-of-flight mass spectrometer (JEOL, Tokyo, Japan) equipped with a source of ions for electron ionization (EI), using a DB-WAX capillary column (0.25 mm×0.25 $\mu$m×30 m) (Agilent, Santa Clara, CA, U.S.A.). The GC conditions were as follows: split injection mode (50 : 1), 1-$\mu$L injection, injection port temperature 230°C, and column oven temperature programmed from 50 to 230°C at 10°C/min. The MS conditions were as follows: electron ionization mode (EI+, ionization energy 70 eV, ionization current 300 $\mu$A), ion source temperature 280°C, and $m/z$ range 29–800. The derivatives of standard monosaccharides were also prepared and analyzed as described above. The identity of each peak was confirmed by comparing its retention time and MS with those of the standards.

## RESULTS

**Isolation and Identification of EPS-Producing Thermophilic LAB** In the present study, 327 LAB candidates were isolated from 37 kinds of tropical fruits taken from 16 provinces of Thailand (Table 1). As a preliminary growth test, although 120 strains of these candidates were observed to grow even at 45°C, it was finally confirmed that 28 strains are thermophilic LAB after the cell viability test. From the result of the nucleotide sequence analysis of the 16S ribosomal RNA-encoding gene, the lychee-derived LY45 strain and the pineapple-derived PY45 strain were identified as *P. pentosaceus* (99.8% identity with *P. pentosaceus* DSM 20336$^T$) and *Lb. amylovorus* (99.6% identity with *Lb. amylovorus* DSM 20531$^T$), respectively. Both of the strains were observed to produce EPSs.

**Effect of Temperature on the Growth of Both LAB Strains** The optimal temperature for growing the LY45 and PY45 strains was investigated (Fig. 1). Both strains grew vigorously at 37 and 45°C. In addition, the pH of the culture broth nearly reached 4 within 24 h. Notably, under a culture condition of 45°C, the pH of the culture broth of both strains rapidly decreased, indicating that the optimal temperature for growing these strains is 45°C, rather than 37°C. It was also observed that these strains can grow at 28 and 50°C, although growth at these temperature was weaker than at 37 and 45°C. However, the pH of the culture broth of the PY45 strain decreased more slowly than did that of the LY45 strain under the given culture temperatures, indicating that the optimal temperature range for growing the PY45 strain is not as broad. Since the observed pH decrease was temporary and reached nearly 5 at 55°C, this temperature is not suitable for growing both strains.

**Sugars Effective for Producing EPSs** The EPS productivity of each strain was examined on a 40-mL culture scale. To determine whether sugar is effective for the high production of EPSs, fructose, galactose, lactose, maltose, mannose, or sucrose was added at a concentration of 1% (w/v) to the modified SDM that contained 2% (w/v) glucose (Table 2). After 2 d of cultivation, adding maltose improved the EPS productivity of the LY45 strain 1.2 fold. In the case of the PY45 strain, after 5 d of culture, the addition of fructose improved the productivity of EPSs 1.1 fold.

We also investigated which concentration of sugar supplemented to the modified SDM when starting cultivation is effective for high EPS productivity on a 40-mL culture scale (Table 3). EPS productivity increased in proportion to the amount of added maltose, indicating that the EPS productivity of the LY45 strain is improved by adding maltose to the culture medium. The addition of up to 5% (w/v) maltose was confirmed to increase EPS productivity. However, since an excessive amount of sugar may induce the Maillard reaction (data not shown), we determined that the necessary concentration of supplemental sugar is 2% (w/v). In addition, supplementation with fructose slightly increased the EPS production of the PY45 strain. However, the yield of EPSs was decreased by adding more than 2% (w/v) sugar.

We also investigated the effect of cultivation time on the EPS production of both strains. The EPS productivity of each strain gradually decreased with the length of the cultivation period. Judging from this result, we determined that the cultivation period should be 2–3 d. In fact, sufficient amounts of EPSs are produced during this period (data not shown).

We expected an increase in EPS productivity on a flask scale (2–5 L) using a modified SDM (Table 3). When the LY45 strain was cultured in 2 L of the medium supplemented with 2% (w/v) maltose, the yield of the purified EPS reached 23 mg/L at 48 h of cultivation. On the other hand, the EPS purified from the culture broth obtained by growing the PY45 strain in the 5-L medium supplemented with 1% (w/v) fructose for 72 h was 6.8 mg/L. The viability of cells of the LY45 or PY45 strain detected in 1 mL of each culture broth was $1.8\times10^8$ or $8.5\times10^7$ CFU, respectively. That is, the EPS yields may not be affected by the growth differences between the strains.

**Inhibitory Effect of EPSs on Hyaluronidase Activity**

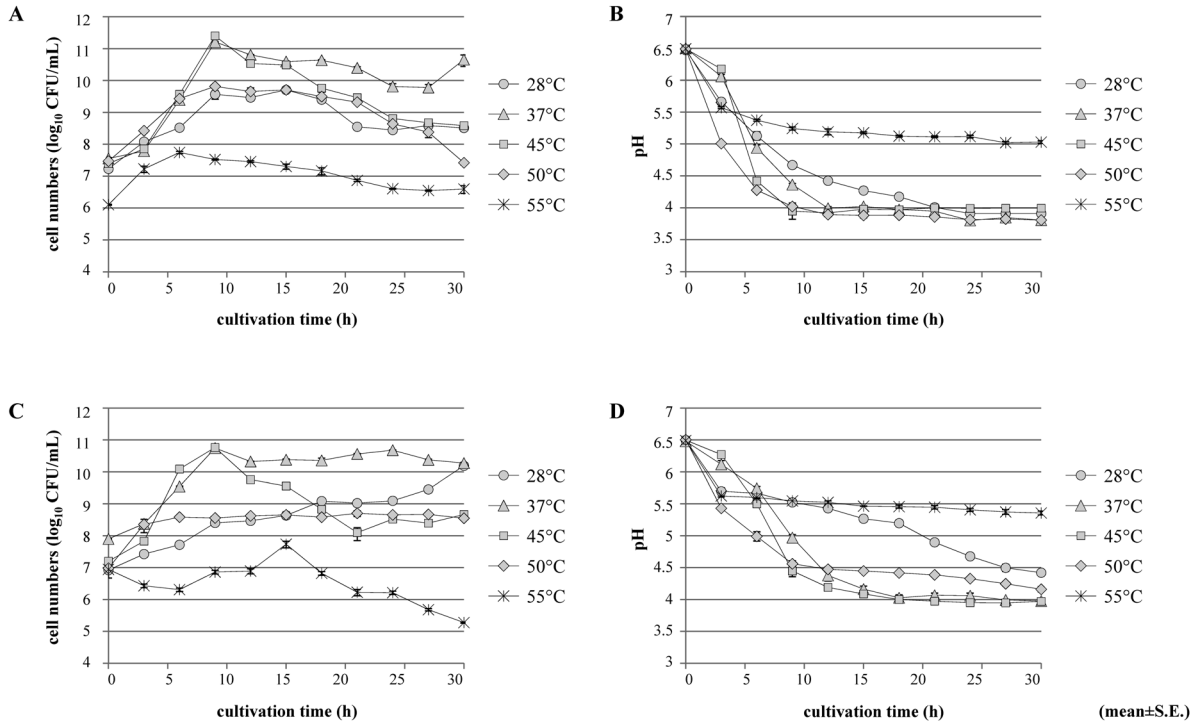


Fig. 1. Effect of Cultivation Temperature on the Cell Growth and pH of the Culture Broth during Cultivation of *P. pentosaceus* LY45 (A and B) or *Lb. amylovorus* PY45 (C and D)

After the inoculation of the seed culture, the number of cells (A and C) and the pH (B and D) of the culture broth were measured at 3-h intervals. The data are indicated as mean±S.E. of three individual experiments.

Table 2. The Effect on the EPS Yield of Added Sugars

| Added sugar | EPS yield % (/control) | |
|---|---|---|
| | LY45 | PY45 |
| - (glucose only) | 100 | 100 |
| Mannose | 94 | 112 |
| Sucrose | 92 | 103 |
| Maltose | 122 | 102 |
| Lactose | 108 | 108 |
| Fructose | 103 | 113 |
| Galactose | 113 | 97 |

The digestive compound of hyaluronic acid by hyaluronidase stimulates inflammation[24,25]; therefore, the anti-inflammatory potentials of EPSs from each of the LY45 and PY45 strains were evaluated by measuring the $IC_{50}$ value for hyaluronidase inhibition (Table 4). Each purified EPS was added to the reaction mixture in various concentrations prior to the hyaluronidase reaction. The $IC_{50}$ values for hyaluronidase inhibition of EPSs purified from LY45 and PY45 strains were 250 and 610 $\mu$g/mL, respectively. When fucoidan, which has been known to inhibit hyaluronidase,[26] was used as a reference for polysaccharide, the inhibitory effect was scarcely observed ($IC_{50}$ may be over 5000 $\mu$g/mL). The $IC_{50}$ value (100 $\mu$g/mL) for sodium cromoglicate, a well-known anti-inflammatory agent, was lower (530 $\mu$g/mL) than that for dipotassium glycyrrhizinate. However, ketotifen fumarate, which is also a known anti-inflammatory agent,[27,28] did not inhibit hyaluronidase activity, even with a concentration of 2000 $\mu$g/mL. Each EPS from LY45 and PY45 strains were further separated into neutral- and acidic-fractions. The $IC_{50}$ value for each acidic EPS was obviously higher than that for neutral one.

**Sugar Composition of EPSs** The anion-exchange column chromatography (TOYOPEARL DEAE-650M) profile indicates that each EPS purified from the culture broth of the LY45 and PY45 strains contains neutral and acidic EPSs; however, the content ratio between the two EPS types is quite different. The LY45-derived EPS contains mainly neutral EPSs (about a 4.8-fold higher yield than that of the acidic EPS), whereas the PY45-derived EPS is composed of both neutral and acidic EPSs with a 1.7 : 1 ratio (Fig. 2).

The GC-MS profile for the LY45-EPS indicates that the neutral EPS consists of glucose and mannose as monosaccharides. On the other hand, the acidic EPS contains mainly mannose, along with glucose and galactose (Fig. 3A). Figure 3B demonstrates that the neutral and acidic EPSs from the PY45-derived EPS are composed mainly of mannose, with only a small amount of glucose.

## DISCUSSION

In recent years, the health role of polysaccharides such as $\beta$-glucan and fucoidan has become a hot topic.[29,30] In the present study, we isolated the EPS-producing thermophilic LAB strains from tropical fruits of Thailand and investigated the health benefits of the EPSs from the LAB strains. As a strategy, it is significant to know whether an EPS exhibits anti-inflammatory properties.

The inhibitory activity of hyaluronidase in inflammatory reactions[31] has been shown to correlate positively with the inhibition of histamine release in anti-allergic assessments by using a chemical mediator release inhibitor, such as sodium cromoglicate or tranilast.[32] Furthermore, natural polysaccha-

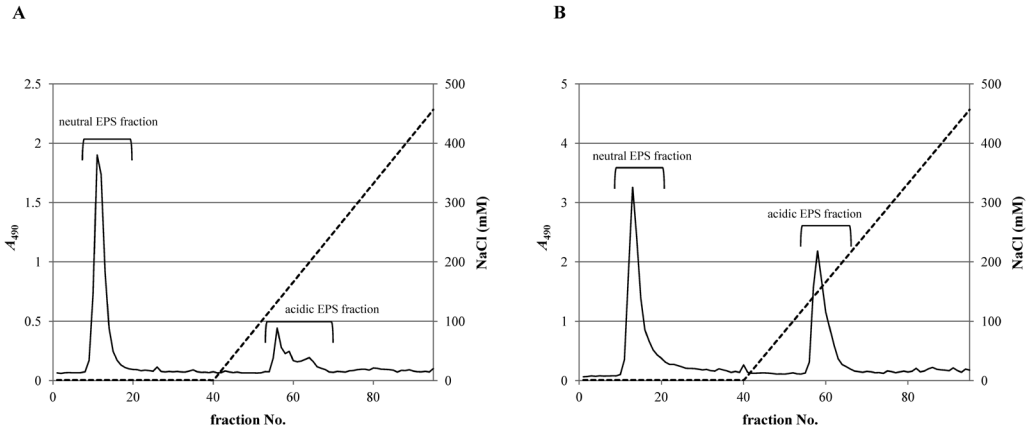


Fig. 2. Fractionation Profiles of the EPS Purified from *P. pentosaceus* LY45 (A) and *Lb. Amylovorus* PY45 (B) by Anion-Exchange Chromatography

TOYOPEARL DEAE-650M Column was used in this study. The EPS eluted in each fraction was monitored at 490 nm by the phenol sulfate method (solid line). Dashed lines indicate the NaCl concentrations in the eluates.

Table 3. The Amount of EPS Purified from Each Culture

| LY45 | EPS yield (mg) from | | PY45 | EPS yield (mg) from | |
|---|---|---|---|---|---|
| Maltose % (w/v) | 40 mL culture | 2 L culture | Fructose % (w/v) | 40 mL culture | 5 L culture |
| 4 | 1.4 | | 4 | 0.22 | |
| 2 | 1.1 | 46 | 2 | 0.23 | |
| 1 | 0.92 | | 1 | 0.26 | 34 |
| 0 | 0.78 | | 0 | 0.23 | |

Table 4. $IC_{50}$ Values for the Hyaluronidase Inhibitory Activities of EPSs and Inhibitors

| EPSs and inhibitors | $IC_{50}$ ($\mu$g/mL) |
|---|---|
| EPS from LY45 (before separation) | 250 |
| Neutral EPS | 380 |
| Acidic EPS | 1300 |
| EPS from PY45 (before separation) | 610 |
| Neutral EPS | 1660 |
| Acidic EPS | 2000<* |
| Fucoidan (from *Laminaria japonica*) | 2000<* |
| Sodium cromoglicate | 100 |
| Ketotifen fumarate | 2000<* |
| Dipotassium glycyrrhizinate | 530 |

*No inhibitory effect was observed up to 2000 $\mu$g/mL.

rides from marine algae (*Gracilaria lemaneiformis*)[33] and terrestrial plants (*Ganoderma tsugae* and *Angelica sinensis*)[34,35] have been shown to exhibit inhibitory effects against immunoglobulin E (IgE)-mediated mast cell degranulation accompanied by the release of histamine.

Some strains of *Bifidobacterium* sp. and *Lb. rhamnosus* GG have been shown to have the same inhibitory activity.[36] To evaluate whether EPSs produced by the isolated thermophilic LY45 and PY45 strains exhibit anti-allergic activity, we measured the inhibitory activity of each EPS together with the well-known anti-inflammatory agents against hyaluronidase (Table 4). The results indicated that the inhibitory effect of ketotifen fumarate was scarcely observed. Sodium cromoglicate shows pharmacological effect by inhibiting an inflammatory chemical mediator release from mast cell.[32] Dipotassium glycyrrhizinate inhibits the enzyme participated in the production of the inflammatory chemical mediator.[37] On the other hand, ketotifen fumarate shows pharmacological effect by inhibiting histamine receptor.[27,28] Thus, the mode of action is different by each chemical known anti-inflammatory agents. When compared with sodium cromoglicate and dipotassium glycyrrhizinate, the $IC_{50}$ values for the LY45- and PY45-derived EPSs had approximately the same level as these agents, respectively. Sodium cromoglicate, a typical hyaluronidase inhibitor, has been widely used as an anti-inflammatory agent. The EPSs produced by the LY45 and PY45 strains may also be expected to become a new anti-allergic material. Especially, the PY45 strain can utilize starch as a carbon source; therefore, the strain may generate useful EPSs from a biomass starch. Our result also shows that the neutral EPSs inhibit the hyaluronidase activity more effectively than acidic one including fucoidan. This suggests that the inhibitory effect to hyaluronidase is mainly occurred by the neutral EPS. However, the EPS without the separation have lower $IC_{50}$ values than that with the separation, suggesting that the neutral EPS mixed with acidic one may inhibit synergistically. $\beta$-Hexosaminidase release is known to be a marker of mast cell degranulation, and the phenomenon implies that inflammatory mediators, such as histamines, are released from the cell.[38] In the present study, we evaluated the anti-allergic activity of LAB-derived EPSs by $\beta$-hexosaminidase release assay. As a result, we confirmed that the release of $\beta$-hexosaminidase from RBL-2H3 cells decreased dose-dependently in the presence of EPS produced by the LY45 strain (data not shown). This phenomenon suggests that the EPS can inhibit the release of histamines from mast cells.

Significantly, the optimal temperature for the growth of LY45 and PY45 strains is 45°C. Although the growth rate is low, it should be noted that both strains can grow even at 50°C. There have been some reports about a thermophilic *Lb.*

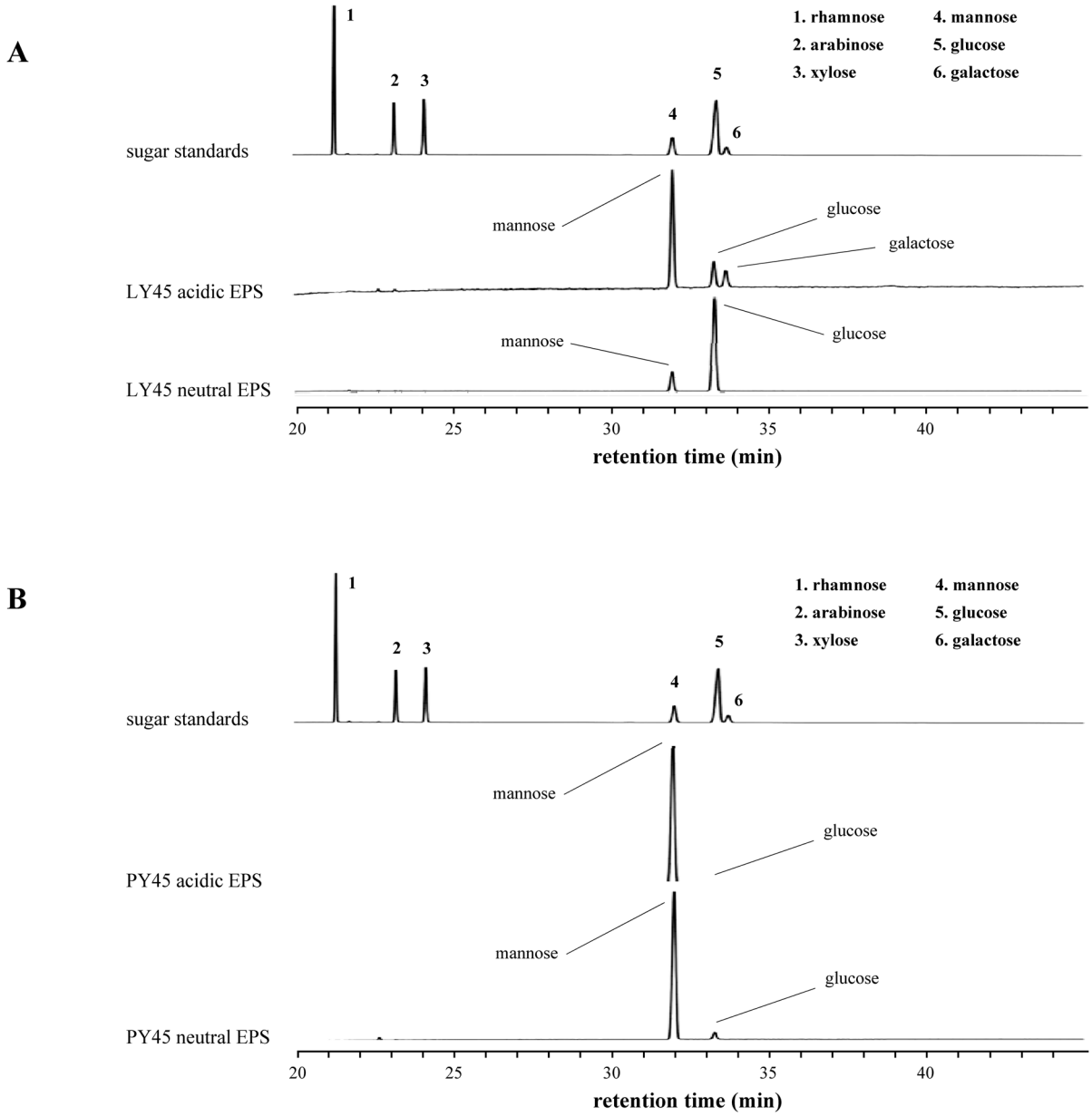


Fig. 3. Chromatographic Profiles of the GC-MS Analyses of EPS Purified from the Culture Broth *P. pentosaceus* LY45 (A) and *Lb. amylovorus* PY45 (B)

The EPS component monosaccharides are detected as alditol acetate derivatives. The identity of each peak was confirmed by its retention time and mass spectrometry.

*amylovorus* strain that was isolated.[23,39,40] The LAB species secretes amylase into the culture broth, suggesting that the LAB species may be useful for making silage. In fact, the PY45 strain that we have identified as *Lb. amylovorus* grows vigorously when starch or glycogen is added as a sole carbon source (data not shown). Although there has been a report that *Lb. amylovorus* produces EPSs,[41] the monosaccharides that constitute the EPS have not yet been analyzed. On the other hand, *P. pentosaceus*, which is often detected in Japanese pickles, can grow even in the presence of up to 10% (w/v) NaCl.[7,8] In fact, the LY45 strain isolated from lychee can also grow at that concentration of NaCl. The LY45 and PY45 strains grew even at 50°C, whereas *P. pentosaceus* LP28, which produces an EPS modified by pyruvate residue,[14] did not (data not shown).

LAB fermentation advances even at low temperatures, causing the flavors and taste of fermented foods to worsen due to the overgrowth of LABs. Since thermophilic LAB strains scarcely grow at lower temperatures, they will be useful in the food industry. In fact, the LY45 and PY45 strains were unable to grow even at 10°C (data not shown).

It has been widely recognized that enzymes from thermophilic organisms are thermostable[42]; for example, a DNA polymerase used in PCR, *Taq* polymerase, is an enzyme found in the hyperthermophilic bacterium *Thermus aquaticus*.[43] In general, mesophilic and psychrophilic enzymes, which originate from mesophilic and psychrophilic microbes, respectively, display optimal activity at normal temperatures; however, these enzymes are inactive at higher temperatures. Therefore, it can be expected that the enzymes from thermophilic LAB strains are thermostable. Information about the molecular biological analysis of EPS-biosynthetic enzymes from ther-

mophilic LAB strains may contribute to a protein engineering design of industrially useful enzymes.

Glucose and galactose are often found to be components of EPSs produced by LAB.[44] The present study shows that these sugars are certainly detected in EPSs produced by the LY45 and PY45 strains, whereas it should be noted that mannose is also detected in a higher ratio. Specifically, the GC-MS profile indicates that the EPS from LY45 and PY45 strains is composed mainly of mannose, except for the neutral EPS produced by the LY45 strain (Fig. 3). The EPS, which contains mannose as a main-component monosaccharide, has been reported only in fungi and yeast.[45–47] Mannose has been reported to be a minor component of the EPSs produced by LAB.[44,48,49] In addition, with respect to LAB-derived EPSs having a high mannose content,[46,50,51] the mannose content in the EPS produced by *Lb. mucosae* DPC 6426, *Lb. plantarum* MTCC 9510, or *Pediococcus* sp. MR17 is 43, 30, or 33%, respectively. Only the strain *Lactococcus lactis* ssp. *lactis* B-6, which was isolated from the traditional South Asian fermented milk, dahi, as a mesophile, has been reported to produce an EPS with a very high mannose content (88%).[52] A soluble fiber found in some plants, glucomannan, is also known as a high-mannan-content polysaccharide. It has been reported that the polysaccharide produces some health benefits related to lowering serum cholesterol, triglycerides, fasting blood glucose, body weight, and so on.[53]

Although further functionality characterization of the high-mannose-content EPSs produced by LY45 and PY45 strains is an urgent problem, both strains may be useful as a meaningful microbial resource in the healthcare industry.

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