Exopolysaccharide Produced by Plant-Derived Lactobacillus plantarum SN35N Exhibits Antiviral Activity

Masafumi Noda,a Narandalai Danshiitsoodol,a Takemasa Sakaguchi,b Keishi Kanno,c and Masanori Sugiyama*a,c

a Department of Probiotic Science for Preventive Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan; b Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan; and c Department of Gastroenterology, Hiroshima University Hospital; 1–2–3 Kasumi, Minami-ku, Hiroshima 734–8551, Japan.

Received June 19, 2021; accepted September 7, 2021

A lactic acid bacterial strain, Lactobacillus plantarum SN35N, which has been isolated from the pear, secretes negatively charged acidic exopolysaccharide (EPS) to outside cells. We have previously found that the SN35N-derived acidic EPS inhibits the catalytic activity of hyaluronidase (EC 3.2.1.35) promoting inflammation. The aim of this study is to find other health benefits of EPS. EPS has been found to exhibit an inhibitory effect against the influenza virus (Alphainfluenzavirus Influenza A virus) and feline calicivirus (Vesivirus Feline calicivirus), which is recognized as a model of norovirus. Although more studies on the structure–function relationship of EPSs are needed, SN35N-derived EPS is a promising lead for developing not only anti-inflammatory agents, but also antiviral substances.

Key words Lactobacillus plantarum; lactic acid bacteria; exopolysaccharide; antivirus

INTRODUCTION

When administered in adequate amounts, living microorganisms that confer health benefits to the host are called “probiotics.” Lactic acid bacteria (LABs), as typical probiotics, have been used to produce fermented dairy products and other fermented foods. “LAB” is a generic name of non-pathogenic Gram-positive bacteria that generate one or two moles of lactic acid from one mole of sugar by fermentation. Some LAB strains have been reported to have potent health benefits with regard to immune modulation and lifestyle-related diseases. LAB strains are roughly classified into two groups based on their isolation sources. One group consists of strains isolated from dairy products and intestines, and the other includes isolates from plant sources, such as vegetables, fruits, flowers, and medicinal herbs. The former and the latter are called animal- and plant-derived LABs, respectively. Because plant-derived LABs are generally more resistant to harsh environments than animal-derived LABs, we were interested in characteristics of plant-derived LABs.

We have already isolated and identified more than 1000 strains of LABs from plant sources and found that some of them are useful in preventive medicine, such as immune modulation, anti-obesity, and anti-inflammation medications. We have previously isolated some exopolysaccharide (EPS)-producing plant-derived LAB strains. These EPSs have been reported to inhibit the enzymatic activity of hyaluronidase (EC 3.2.1.36), which promotes inflammatory reactions. The hyaluronidase-inhibitory activity has been reported to correlate with histamine-release inhibition in inflammatory reactions through immunoglobulin E (IgE)-mediated mast cell degranulation. Therefore, EPSs that display hyaluronidase-inhibitory activity may have preventive and restorative effects against inflammatory diseases. In fact, EPS produced by the IJH-SONE68 strain has been demonstrated to prevent and improve picryl chloride-induced contact dermatitis in model mice.

The aim of this study is to investigate EPS to find other health-promoting functions. During the experiment, we found that negatively charged acidic EPS produced by the pear-derived Lactobacillus (Lb.) plantarum SN35N inhibits infection of influenza virus (Alphainfluenzavirus Influenza virus A (IFV A)) and feline calicivirus (Vesivirus Feline calicivirus (FCV)), as cultivable surrogates for norovirus (Norovirus Norwalk virus), to the corresponding cultured cells. IFV A, which is the predominant virus in the seasonal flu, often causes pandemic outbreaks. Norovirus infection causes sudden severe vomiting and diarrhea if the outbreak occurs in public facilities. Because norovirus cannot be cultured in the laboratory, FCV is generally used as an alternative virus due to their relatedness and similarity in size.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions Lb. plantarum SN35N and Lb. amylovorus PY45, which our research group isolated previously, were used as EPS-producing LAB strains. De Man, Rogosa, and Sharpe (MRS) medium (Merck KGaA, Darmstadt, Germany) was used for the seed cultivation of LAB strains. A modified semi-defined medium (SDM), which consists of SDM supplemented with 0.2% (v/v) vitamin mixture and 0.1% (v/v) trace element solution instead of yeast nitrogen bases, was used to produce EPS.

Purification of EPS from a Culture Broth of LABs The EPS from LAB strains was purified according to the previous report. Briefly, each seed culture was inoculated at 0.5% (v/v) into a fresh, modified SDM and incubated at 28 or 45°C.
for 2 d to grow SN35N or PY45 strains, respectively. The cultured broth was boiled for 30 min, followed by adding a 100% (w/v) trichloroacetic acid (TCA) solution to the culture broth at a final concentration of 4% (v/v). The cell debris was precipitated by centrifugation, and the culture supernatant was collected. The acetone-precipitated crude EPS obtained by centrifugation was dried up and dissolved into a 50 mM Tris–HCl buffer (pH 8.0). After treatment with nuclease and protease, the EPS was precipitated by adding 3 volumes of ethanol and collected by centrifugation. The precipitant was dried up and dissolved into an appropriate volume of sterile water. Each EPS solution was dialyzed against sterile distilled water by using Amicon Ultra (MWCO = 10kDa, Merck Millipore Ltd., Carrigtwohill, County Cork, Ireland) to obtain a purified EPS mixture.

The neutral and acidic EPSs were further separated by column chromatography using the anion exchange resin (TOYOPEARL DEAE-650M, Tosoh Bioscience, Tokyo, Japan) as described previously.29 After dialysis against sterile distilled water using Amicon Ultra (MWCO = 10kDa), each EPS sample was lyophilized and stored at 4 °C until use. The SN35N-derived acidic EPS has been recently demonstrated to be composed of glucose, galactose, and mannose at a molecular ratio of 15.0 : 5.7 : 1.0, and its molecular weight was estimated approximately 250 kDa.20

**Antiviral Assay** An antiviral assay was performed as described previously.20 MDCK (+) cells (canine kidney-derived cells, described in the previous literature)31 and CRFK cells (feline kidney-derived cells) were infected with IFV A/Udorn/72 (H3N2) and FCV F9 strains, respectively. The cells were incubated in DMEM until cytopathic effects were preliminarily incubated with IFV or FCV for 3 min at room temperature (r.t.), the concentration at which the RBCs appeared cloudy at the bottom of the well was defined as hemagglutination positive, whereas non-agglutinated RBCs made a small red button in the well.

**Neuraminidase-Inhibition Assay** The inhibitory activities against the neuraminidase of EPS samples were evaluated using an EnzyChrom Neuraminidase Assay Kit (BioAssay Systems, Hayward, CA, U.S.A.) with purified neuraminidase enzymes (from *Arthrobacter ureafaciens*, Nacalai Tesque, Inc., Kyoto, Japan). The assay was conducted by a colorimetric procedure in accordance with the manufacturer’s protocol.

### RESULTS

**Antivirus Activities of EPS** The SN35N-derived EPS was preliminarily incubated with IFV or FCV for 3 min at r.t. before exposure to infected cells. This EPS strain significantly inactivated the infectivity of IFV to less than the detection limit by more than a 5-log reduction (Fig. 1). It also significantly inactivated FCV by more than a 3-log reduction. On the other hand, the EPS derived from *L. amylovorus* PY45 did not suppress IFV or FCV infection (Fig. 1). The EPS solutions

![Fig. 1. Antiviral Effects of the SN35N Acidic EPS against Host Cell Infection with IFV and FCV](image-url)
did not show any cytotoxicity at the concentrations used in the test, demonstrating that the SN35N EPS can fully inactivate IFV and FCV, a surrogate of Norwalk virus from the Noro-virus genus. On the other hand, no inhibitory effects were observed against either virus on fucoidan.

**Hemagglutination-Inhibitory Effect of EPS** The influenza virus has a membrane fusion glycoprotein, called hemagglutinin, which causes a virus to bind to target cells through interaction with sialic acid. Hemagglutination assay was used to evaluate this virus binding to facilitating host cells to determine whether LAB-derived EPS inhibits the virus–target cell interaction. In the presence of 50 ng/mL INF A antigen, the sheep RBCs were significantly agglutinated (Fig. 2). A control EPS, which does not have antiviral activity but inhibits IFV A antigen-mediated hemagglutination, showed inhibition activity in a dose-dependent manner. However, SN35N EPS could not inhibit hemagglutination.

**Neuraminidase-Inhibition Assay** The influenza virus’s membrane fusion enzyme, neuraminidase, also known as sialidase, plays several important roles in infection: 1) promoting the release of virus from infected cells, 2) helping the virus itself to spread to uninfected cells nearby, and 3) preventing the aggregation of the virus. We confirmed whether the SN35N EPS affected the activity of the neuraminidase; however, no inhibition was observed (data not shown).

**DISCUSSION**

The relationship between bioactivity and the chemical structure of EPS is not elucidated at this time. Therefore, more information with regard to the relationship of EPSs from other LABs is needed to clarify the problem. It has been reported that the antimicrobial properties of the LAB strain, including antiviral effects, result from its immune-modulating property. However, EPS produced by animal-derived *Lb. delbrueckii* ssp. *bulgaricus* OLL1073R-1 has been reported to exhibit anti-IFV activity by augmenting the activity of natural killer cells. Unlike in the case of the OLL1073R-1 strain, the antiviral effect of the SN35N-derived EPS was observed in vitro, suggesting that EPS may bind to the virus surface directly without hemagglutinin and neuraminidase, which are important for IFV infection, and may inhibit the binding of the virus to host cells. Therefore, it might be predicted that EPS interacts non-specifically with unknown targets of the virus, with the result that adhesion of the virus particle to the target cell may be inhibited.

In contrast to the hyaluronidase inhibition, the antiviral activity of EPSs seems not to be widely preserved in EPSs. In fact, the PY45-derived EPS has no effect on virus infection (Fig. 1). We also analyzed the antiviral activity of EPSs obtained from other LABs and sulfated polysaccharide fucoidan, but no remarkable effects were observed (data not shown). Spirulan, which is a specific sulfated acidic polysaccharide composed of some saccharides, sulfate, and calcium, is extracted from a blue–green alga, *Spirulina platensis*. Hayashi et al. showed that spirulan inhibits the replication of several enveloped viruses by interfering with the interaction between viruses and host cells; however, chelation of the calcium ion with sulfate groups results in the loss of antiviral activity. Generally, virus infection is initiated by a virus–host cell binding mediated by a virus surface glycoprotein, such as hemagglutinin proteins, and glycan receptors on the surface of the host cell. The viral glycan receptors contain sialic acid and thus have a negative charge, whereas hemagglutinin proteins tend to charge positively. This virus–host cell binding might play an important role in not only viral attachment but also its entry into host cells. Therefore, the negatively charged acidic polysaccharides seem to be predicted to interact with the positively charged receptors, resulting in the inhibition of virus attachment and invasion of host cells. In fact, a sulfated acidic EPS, heparan sulfate, has also been found to bind with the virus surface glycoprotein and to inhibit the interaction between virus and cognate receptor competitively. However, no antiviral activities were observed on fucoidan in this study, in spite of its sulfate residue. Therefore, the antiviral activity found on SN35N-derived EPS is expected to be due to its sulfate content.
to its unique structural characteristics rather than only its acidic properties. Our previous report showed that the SN35N strain has four EPS-biosynthesizing gene clusters on its chromosomal DNA, and another one is located on the pSN35N-3 plasmid. Interestingly, although the EPS productivity of the plasmid pSN35N-3-cured mutant (SN35N-Δp3) was drastically decreased (from 48.2 to 3.3 mg/L), our preparatory experiments indicated that the EPS obtained from SN35N-Δp3 is more effective against IFV infection (data not shown). This result suggests that the EPS clusters on chromosomal DNA are more important in the anti-viral activity but not in the production of EPS.

This study reports inhibitory effects observed in the *Lb. plantarum* SN35N-derived EPS on virus infection of host cells. Although more studies on the structural characterization and structure–function relationship of EPSs are needed, EPSs show promise for developing not only anti-inflammatory agents but also antiviral substances.

Acknowledgments We are grateful to Marie Mizuguchi and Nasrin Sultana for their technical assistance in the Sugiya laboratory. We also thank the Analysis Center of Life Science, Hiroshima University, for the use of their facilities.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Sanders ME, Probiotics: definitions, sources, selection, and uses. *Clin. Infect. Dis.*, **46** (Suppl. 2), S58–S61, discussion, S14–S151 (2008).

2) Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.*, **5**, 77–78 (2005).

3) Adolfsson O, Meydani SN, Russell RM. Yogurt and gut function. *Am. J. Clin. Nutr.*, **80**, 245–256 (2004).

4) Cross ML, Stevenson LM, Gill HS. Anti-allergy properties of fermented foods: an important immunoregulatory mechanism of lactic acid bacteria? *Int. Immunopharmacol.*, **1**, 891–901 (2001).

5) Heyman M. Effect of lactic acid bacteria on diarrheal diseases. *J. Appl. Microbiol.*, **99** (Suppl. 1), 137S–146S (2000).

6) Meydani SN, Ha WK. Immunologic effects of yogurt. *Am. J. Clin. Nutr.*, **71**, 861–872 (2000).

7) Parvez S, Malik KA, Ab Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. *J. Appl. Microbiol.*, **100**, 1171–1185 (2006).

8) Wang Y, Xu N, Xi A, Ahmed Z, Zhang B, Bai X. Effects of *Lactobacillus plantarum* MA2 isolated from Tibetan kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Appl. Microbiol. Biotechnol.*, **84**, 341–347 (2009).

9) Nguyen TD, Kang JH, Lee MS. Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacteria with cholesterol-lowering effects. *Int. J. Food Microbiol.*, **113**, 358–361 (2007).

10) Liong MT, Shah NP. Effects of *Lactobacillus casei* synbiotic on serum lipoprotein, intestinal microflora, and organic acids in rats. *J. Dairy Sci.*, **89**, 1390–1399 (2006).

11) Lavermicocca P, Valerio F, Lonigro SL, De Angelis M, Morelli L, Callegari ML, Rizzello CG, Visconti A. Study of adhesion and survival of lactobacilli and bifidobacteria on table olives with the aim of formulating a new probiotic food. *Appl. Environ. Microbiol.*, **71**, 4233–4240 (2005).

12) Okada S. The world of plant origin lactic acid bacteria. *Jpn. J. Lactic Acid Bacteria*, **13**, 23–36 (2002).

13) Van Hylckama Vlieg JE†, Rademaker JLW, Bachmann H, Molenaar D, Kelly WJ, Siezen RJ. Natural diversity and adaptive responses of *Lactococcus lactis*. *Curr. Opin. Biotechnol.*, **17**, 183–190 (2006).

14) Jin H, Higashikawa F, Noda M, Zhao X, Matoba Y, Kumagai T, Sugiyama M. Establishment of an in vitro Peyser’s patch cell culture system correlative to in vivo study using intestine and screening of lactic acid bacteria enhancing intestinal immunity. *Biol. Pharm. Bull.*, **33**, 289–293 (2010).

15) Zhao X, Higashikawa F, Noda M, Kawamura Y, Matoba Y, Kumagai T, Sugiyama M. The obesity and fatty liver are reduced by plant-derived *Pediococcus pentosaceus* LP28 in high fat diet-induced obese mice. *PLoS ONE*, **7**, e30692 (2012).

16) Higashikawa F, Noda M, Awa T, Danhiisootdool N, Matoba Y, Kumagai T, Sugiyama M. Antiobesity effect of *Pediococcus pentosaceus* LP28 on overweight subjects: a randomized, double-blind, placebo-controlled clinical trial. *Eur. J. Clin. Nutr.*, **70**, 582–587 (2016).

17) Okamoto T, Sugimoto S, Noda M, Yokooji T, Danhiisootdool N, Higashikawa F, Sugiyama M. Interleukin-6 release inhibitors generated by fermentation of *Artemisia princeps* Pampamini herb extract with *Lactobacillus plantarum* SN13T. *Front. Microbiol.*, **11**, 1159 (2020).

18) Panthavee W, Noda M, Danhiisootdool N, Kumagai T, Sugiyama M. Characterization of exopolysaccharides produced by thermophilic lactic acid bacteria isolated from tropical fruits of Thailand. *Biol. Pharm. Bull.*, **40**, 621–629 (2017).

19) Noda M, Sugimoto S, Hayashia T, Danhiisootdool N, Fukamachi M, Sugiyama M. A novel structure of exopolysaccharide produced by a plant-derived lactic acid bacterium *Lactobacillus paracasei* IJH-SONE68. *J. Biochem.*, **164**, 87–92 (2018).

20) Noda M, Shiraga M, Kumagai T, Danhiisootdool N, Sugiyama M. Characterization of the SN35N strain-specific exopolysaccharide encoded in the whole circular genome of a plant-derived *Lactobacillus plantarum*. *Biol. Pharm. Bull.*, **41**, 536–545 (2018).

21) Kakegawa H, Matsumoto H, Sato T. Activation of hyaluronidase by metallic salts and compound 48/80, and inhibitory effect of anti-allergic agents on hyaluronidase. *Chem. Pharm. Bull.*, **33**, 642–646 (1985).

22) Fujitani N, Sakaki S, Yamaguchi Y, Takenaka H. Inhibitory effects of microbialase on the activation of hyaluronidase. *J. Appl. Physiol.*, **13**, 489–492 (2001).

23) Maeda Y, Yamamoto M, Masui T, Sugiyama K, Yokota M, Naka gomi K, Tanaka H, Takahashi I, Kobayashi T, Kobayashi E. Inhibitory effect of tea extracts on hyaluronidase: studies on anti-allergic activity in tea. *II. Shokuhin Eiseigaku Zasshi*, **31**, 233–237 (1990).

24) Noda M, Sultana N, Hayashi I, Fukamachi M, Sugiyama M. Exopolysaccharide produced by *Lactobacillus paracasei* IJH-SONE68 prevents and improves the picrot chloride-induced contact dermatitis. *Molecules*, **24**, 2970 (2019).

25) Bao GR, Chittaganpitch M, Kanai Y, Li YG, Auwanit W, Ikuta K, Sawapanyalert P. Amantadine- and oseltamivir-resistant variants of influenza A viruses in Thailand. *Biochem. Biophys. Res. Commun.*, **390**, 897–901 (2009).

26) Robilotti E, Derenski S, Pinsky BA. Norovirus. *Clin. Microbiol. Rev.*, **28**, 134–164 (2015).

27) Kimmel SA, Roberts RF. Development of a growth medium suitable for exopolysaccharide production by *Lactobacillus delbrueckii* spp. *bulgaricus* RR. *Int. J. Food Microbiol.*, **80**, 87–92 (1998).

28) Kets EPW, Galinski EA, de Bont JAM. Carnitine: a novel compatible solute in *Lactobacillus plantarum*. *Arch. Microbiol.*, **162**, 243–248 (1994).

29) Acosta JC, O’Loghlen A, Banito A, Guirarro MV, Augert A, Raguz S, Fumagalli M, da Costa M, Brown C, Popov N, Takatsy U, Melamed J, d’Adda di Fagagna F, Bernard D, Hernando E, Gil J. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*, **133**, 1006–1018 (2008).

30) Oeda K, Kawabata R, Irie T, Nakai Y, Tohya Y, Sakauchi T, In-
activation of pathogenic viruses by plant-derived tannins: strong effects of extracts from persimmon (*Diospyros kaki*) on a broad range of viruses. *PLOS ONE*, 8, e55343 (2013).

31) Noma K, Kiyotani K, Kouchi H, Fujii Y, Egi Y, Tanaka K, Yoshida T. Endogenous protease-dependent replication of human influenza viruses in two MDCK cell lines. *Arch. Virol.*, 143, 1893–1909 (1998).

32) Hidalgo-Cantabrana C, López P, Gueimonde M, de Los Reyes-Gavilán CG, Suárez A, Margolles A, Ruas-Madiedo P. Immune modulation capability of exopolysaccharides synthesized by lactic acid bacteria and bifidobacteria. *Probiotics Antimicrob. Proteins*, 4, 227–237 (2012).

33) Makino S, Ikegami S, Kano H, Sashihara T, Sugano H, Horiiuchi H, Saito T, Oda M. Immunomodulatory effects of polysaccharides produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *J. Dairy Sci.*, 89, 2873–2881 (2006).

34) Makino S. Immunostimulatory effects of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 and its exopolysaccharides. *Milk Science*, 58, 35–40 (2009).

35) Hayashi T, Hayashi K, Maeda M, Kojima I. Calcium spirulan, an inhibitor of enveloped virus replication, from a blue-green alga *Spirulina platensis*. *J. Nat. Prod.*, 59, 83–87 (1996).

36) Raman R, Tharakaraman K, Sasisekharan V, Sasisekharan R. Glycan-protein interactions in viral pathogenesis. *Curr. Opin. Struct. Biol.*, 40, 153–162 (2016).

37) Arinaminpathy N, Grenfell B. Dynamics of glycoprotein charge in the evolutionary history of human influenza. *PLoS ONE*, 5, e15674 (2010).

38) Kobayashi Y, Suzuki Y. Compensatory evolution of net-charge in influenza A virus hemagglutinin. *PLOS ONE*, 7, e40422 (2012).

39) Mathieu C, Dhondt KP, Châlons M, Mély D, Raoul H, Negre D, Cosset FJ, Gerlier D, Vivès RR, Horvat B. Heparan sulfate-dependent enhancement of henipavirus infection. *MBio*, 6, e02427-14 (2015).