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Full Paper

α-ketoglutarate produced by lactic acid bacteria inhibits hyaluronidase activity

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In Japan, the growing interest in anti-aging skin care is associated with the unprecedented aging society. Skin aging can be attributed to various factors, including the activation of hyaluronidase enzyme in subcutaneous tissues exposed to ultraviolet radiation. This enzyme breaks down hyaluronic acid, leading to skin sagging. Therefore, hyaluronidase inhibitors can effectively prevent skin aging. Previously, food components have been actively explored to search for hyaluronidase inhibitors considering the high safety of these materials. Although lactic acid bacteria (LAB)-fermented foods inhibit this enzyme, their active compounds responsible for hyaluronidase inhibition remain unknown. Thus, in this study, we aimed to explore the mechanism underlying the LAB-mediated inhibition of hyaluronidase activity. Supernatants of a LAB-fermented milk-based beverage were subjected to a hyaluronidase inhibition assay, followed by purification and separation using hydrophobic adsorbents and high-performance liquid chromatography, respectively. Subsequently, liquid chromatograph time-of-flight mass analysis was performed, revealing α-ketoglutarate (AKG) as the inhibitor of this enzyme. The half-maximal inhibitory concentration (IC_{50}) of AKG was approximately 0.13-fold that of the known strong hyaluronidase inhibitor disodium cromoglycate (DSCG). To the best of our knowledge, this is the first report on hyaluronidase inhibition mediated by AKG, a metabolic product of LAB. Additionally, *Lactobacillus acidophilus* JCM1132 was identified as a highly effective AKG-producing LAB (63.9 μg/mL) through LC-MS/MS-based quantitative analyses using various LAB-fermented milk samples. We anticipate that the findings of this study will potentially support the development of functional foods and cosmetics enriched with AKG.

Key words: *Lactobacillus*, *Streptococcus*, hyaluronidase, α-ketoglutarate, skin, anti-aging, fermented milk

INTRODUCTION

The visible signs of skin aging reflect the aging process of individuals. Recently, owing to the increasing older adult population in Japan, a wide variety of products exhibiting anti-aging effects have become widespread in the market. A wide range of products, from cosmetics to functional foods, used as skin-antiaging solutions can potentially draw the interest of consumers.

Two major types of aging processes contribute to skin aging: intrinsic aging, a natural process that occurs with age, and extrinsic aging induced by external factors, such as exposure to direct sunlight, leading to the excessive generation of reactive oxygen species (ROS) in skin tissues and triggering various oxidative stress responses [1]. The mechanism of ultraviolet (UV) radiation-induced ROS production varies with wavelength; for instance, UVB (medium wavelength) reaches the epidermis and induces the production of $O_2^{\bullet-}$ by activating nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase. UVA (long-wavelength) penetrates deeper into the dermis than UVB. In addition to $O_2^{\bullet-}$ production via NADPH oxidase activation, UVA generates $^1O_2$ within cells through chromophores such as riboflavin [2, 3]. These two types of UV induce the production of ROS through the abovementioned mechanisms and trigger an inflammatory response.

The inflammatory response increases the production and activation of the enzyme hyaluronidase, which breaks down hyaluronic acid. This process helps maintain skin moisture but is associated with the appearance of face lines and wrinkles [4, 5]. Hence, ongoing research associated with the development of cosmetic products is focused on the inhibition of this enzyme activity [6, 7]. For example, *Sanguisorba officinalis* root, which inhibits hyaluronidase activity, reduces the appearance of human facial wrinkles [5].

While developing cosmetic products, safety should be prioritized considering the direct application on the human body.

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Consequently, there is a growing need for research on beauty-enhancing ingredients derived from food materials. Several studies have reported the anti-hyaluronidase effects of food components such as phosphoproteins derived from egg yolk [8], seaweeds [9], and fruits [10]. Moreover, the cosmetic benefits of lactic acid bacteria (LAB)-fermented milk have been reported; for instance, milk-derived components, such as sphingomyelin, can help prevent disruption of skin barrier function after UV-B irradiation [11]. The intake of LAB-fermented milk maintains healthy skin in young women [12]. In mice, orally administered fermented milk with *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1247 and *Streptococcus thermophilus* 3078 strains improve the balance of Th-1 and Th-2 cytokines in skin exposed to UV radiation, promote DNA repair, and effectively reduce erythema [13]. Additionally, metabolomic research has revealed that some strains of lactobacilli and streptococci can produce hyaluronic acid [14, 15] and that LAB-derived nicotinamide might protect skin from photoaging by stabilizing mitochondrial function and reducing ROS generation [16]. These reports reveal the beneficial effects of LAB on the external appearance of skin by affecting the gut microbiota, producing cosmetic ingredients, and improving immunity. However, the relationship between LAB and hyaluronidase, which is a key factor in skin aging, is not well understood; only strain-specific metabolites, such as extracellular polysaccharides derived from *Lactiplantibacillus plantarum* SN35N and *Lacticaseibacillus paracasei* IJH-SONE68 [17, 18], have been reported as inhibitors of hyaluronidase activity. Therefore, further research on LAB-derived hyaluronidase inhibitors is required to comprehensively understand the relationship between skin health and LAB and develop new cosmetic products.

In this study, we aimed to explore the active component associated with the inhibition of hyaluronidase activity in a milk-based beverage fermented with LAB. Moreover, we conducted comprehensive analyses to identify the LAB strain that effectively inhibits hyaluronidase activity.

MATERIALS AND METHODS

Chemicals and materials

The fermented milk-based beverage used in this study was provided by Nissin York Co., Ltd. (Tokyo, Japan); it is prepared using *L. paracasei* NY1301, skimmed milk, and sugar. De Man, Rogosa, and Sharpe (MRS) medium was obtained from Biokar Diagnostics (Beauvais, France). Hyaluronidase from bovine testes and compound 48/80 were obtained from Sigma-Aldrich (St. Louis, MI, USA). Acetate, calcium chloride, sodium hyaluronate from rooster comb, boric acid, p-dimethylaminobenzaldehyde, orotic acid, 2-hydroxyglutarate, uracil, methionine, α-ketoglutarate (AKG), nicotinic acid, acetylcarnitine, cadaverine, formic acid, ammonium formate, and disodium cromoglycate (DSCG) were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan). Acetonitrile and Tris were obtained from Kanto Chemical (Tokyo, Japan).

Separation and purification of the fermented milk-based beverage using XAD4

Two hundred grams of the supernatant obtained by centrifuging the fermented milk-based beverage at 10,000 × g (4°C) for 5 min was vacuum filtered and passed through adsorption resin XAD4 (Organo, Tokyo, Japan). The fractions were eluted using 25%, 50%, and 100% ethanol. Each fraction was evaporated, lyophilized, and dissolved to a concentration 20 times higher than the original solution in 10 mL of water.

Hyaluronidase inhibition assay

This assay was conducted following the method described by Maeda *et al.* [19]. Briefly, 5 µL of 10 mg/mL hyaluronidase in 0.1 M acetate buffer (pH 4.0) and 10 µL of either the purified fermented milk-based beverage or distilled water (control) were mixed and incubated at 37°C for 20 min. Ten microliters of 25 mM calcium chloride and 10 µL of 2 mg/mL compound 48/80 were added to the mixture, which was then incubated for 20 min at 37°C. Subsequently, 25 µL of 1 mg/mL sodium hyaluronate was added, and the solution was incubated at 37°C for 40 min. To adjust the final mixture, 10 µL of 0.4 N NaOH and 1.6 M boric acid (pH 9.1) were added, and the mixture was heated at 95°C for 3 min. After cooling to 37°C, 600 µL of p-DAD reagent (a solution prepared by mixing 5 g of p-dimethylaminobenzaldehyde in 6 mL of 10 N HCl followed by 10-fold dilution using acetic acid) was added. After incubation for 20 min at 37°C, 200 µL of the sample solution was collected, and the absorbance was measured at 600 nm using a microplate reader (Infinite F50; Tecan Group AG, Männedorf, Switzerland). The rate of hyaluronidase activity inhibition (%) was calculated as follows:

$$\text{Hyaluronidase activity inhibition rate (\%)} = [1 - (S / C)] \times 100,$$

where S and C are the absorbance of the samples and controls corrected with blanks, respectively. Blanks were samples without hyaluronidase.

Separation and purification of the fermented milk-based beverage using high-performance liquid chromatography (HPLC)

The purified fermented milk-based beverage fractions that exhibited a strong inhibitory effect on hyaluronidase were further separated using HPLC (Shimadzu, Kyoto, Japan) with two columns. The first purification was performed using a large-scale column (InertSustain C18, 10 mm × 250 mm, 5 µm; GL Science, Tokyo, Japan) at 25°C. The sample was eluted with water/acetonitrile (ACN) at a flow rate of 5.0 mL/min and monitored with a UV spectrophotometer at 210 nm. The elution was performed in isocratic mode (2% ACN, 10 min). The second purification was performed using a small-scale column (InertSustain C18, 4.6 mm × 250 mm, 5 µm; GL Science) at 40°C. The sample was eluted with ACN/0.1% formic acid (FA) at a flow rate of 1.0 mL/min and monitored with a UV spectrophotometer at 195 nm. Gradient elution was programmed as follows: 0–1% ACN, 0–10 min; 1–2% ACN, 10–12 min; 2% ACN, 12–15 min; 2–10% ACN, 15–20 min; and 10% ACN, 20–30 min. Each fraction was evaporated, lyophilized, and dissolved in the same amount of water as the volume of the sample loaded into HPLC. Finally, a hyaluronidase inhibition assay was conducted.

Analysis of candidate hyaluronidase inhibitors using liquid chromatography time-of-flight mass spectrometry (LC/TOF-MS)

Separation of fractions was performed using an InertSustain C18 HPLC column (4.6 mm × 250 mm, 5 µm; GL science). The

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fraction showing a strong hyaluronidase inhibitory effect was collected and analyzed using LC/TOF-MS (Shimadzu, Kyoto, Japan). LC separation of the sample was performed using an X-Bridge Amide (2.1 mm × 150 mm, 3.5 µm; Waters, Milford, MA, USA) at 40°C. The sample was eluted with ACN containing 0.1% FA/0.1% FA at a flow rate of 0.25 mL/min and monitored with a UV spectrophotometer at 195 nm and 254 nm. Gradient elution was programmed as follows: 95% ACN containing FA, 0–2 min; 95–20% ACN containing FA, 2–15 min; 20% ACN containing FA, 15–17 min. TOF-MS analysis was conducted on a Bruker impactII QTOF mass spectrometer (Bruker Japan, Kanagawa, Japan) in the electrospray ionization electrospray ionization (ESI)-positive and ESI-negative modes. MS parameters were as follows: drying gas (N_2), 7.0 L/min; drying temperature, 200°C; nebulizer pressure, 2.0 bar; capillary voltage, 3,000 V; and mass range, 50–1,200 *m/z*. The mass spectra of the obtained peaks from the sample measurements were analyzed using Bruker HMDB Metabolite Library 2.0 (Bruker Japan) and Bruker MetaboBASE® Personal Library 3.0 (Bruker Japan). We identified eight candidate compounds as hyaluronidase inhibitors. Subsequently, seven of the eight candidate compounds were diluted in water to prepare 20 mM aqueous solutions, and their anti-hyaluronidase activity was analyzed by the method described above, that is, adding 10 µL of each aqueous solution instead of the purified fermented milk-based beverage. Orotic acid, one of the eight candidate compounds, was insoluble in water; therefore, it was dissolved in 0.1 M Tris-HCl buffer (pH 8.0) and diluted to prepare the 20 mM aqueous solution.

Analysis of the inhibitory effects of AKG on hyaluronidase

AKG and the strong hyaluronidase inhibitor DSCG were used to determine the inhibitory effects on hyaluronidase. AKG was diluted in water to prepare solutions at concentrations of 5, 12, 14, 17, and 20 mM, whereas DSCG was diluted in water to prepare solutions at concentrations of 1, 1.5, 2, 5, and 20 mM. Their anti-hyaluronidase activity and inhibition rates were analyzed using the method described above. The IC_{50}, which indicates the concentration required for 50% inhibition of hyaluronidase, was calculated using the mean of 3–4 measurements.

Bacterial strains and culture conditions

Several LAB strains, including *Lactobacillus acidophilus* JCM1132, *S. thermophilus* NY, *L. paracasei* JCM1109, *L. paracasei* NY1301, *Lacticaseibacillus rhamnosus* ATCC7469, *Enterococcus hirae* ATCC8043, and *Lactobacillus gasseri* JCM1131, were used in this study. Japan Collection of Microorganisms (JCM) strains were obtained from the Japan Collection of Microorganisms (Tsukuba, Japan). NY strains were from Nissin York Co., Ltd. (Tokyo, Japan). ATCC strains were from the American Type Culture Collection (Manassas, VA, USA). All LAB strains were precultured in MRS medium at 37°C for 24 hr.

Milk fermentation

Milk was fermented under the following standard conditions: an MRS-based culture of *L. paracasei* NY1301 was inoculated in the 10% (w/v) skim-milk medium containing 0.3% (w/v) yeast extract to achieve an OD_{600} of 0.001 and precultured at 37°C for 24 hr. Subsequently, the precultured skim-milk medium was added (0.3% v/v) to the 15% (w/v) skim-milk medium containing 10% (w/v) sugar and then cultured. Sample cultures were collected at 0 and 24 hr.

Culture conditions for screening high-level AKG-producing LAB

To screen the strain that produces AKG in milk with high efficiency, each LAB strain was inoculated in the skim-milk medium containing yeast extract to achieve an OD_{600} of 0.001 and pre-cultured at 37°C for 24 hr. Subsequently, the precultured milk medium was added (0.3% v/v) to the yeast extract-supplemented fresh skim-milk medium and further cultured at 37°C for 48 hr.

Quantification of AKG using LC/MS/MS

The supernatant of the fermented milk centrifuged at 10,000 × g (4°C) for 10 min was collected, passed through filter paper, and analyzed using LC/MS/MS. LC/MS/MS was performed using standard samples of AKG dissolved in distilled water to obtain solutions at concentrations of 0.2, 0.5, 1, 5, 7.5, 10, and 20 µg/mL and milk-derived samples. LC separation of the 2 µL sample was performed using a column (Intrada Organic Acid, 2 mm × 150 mm, 3 µm; Imtakt, Kyoto, Japan) at 40°C. The sample was eluted with 10% ACN-water-0.1% FA (buffer A) and 10% ACN/90 mM ammonium formate (buffer B) at a flow rate of 0.25 mL/min. Gradient elution was programmed as follows: 0% buffer B, 0–1 min; 0–100% buffer B, 1–10 min; and 100% buffer B, 10–12 min. MS/MS analysis was conducted on an AB SCIEX QTRAP 6500 spectrometer (SCIEX, MA, USA) in the ESI-negative mode. The source conditions were a standard set up of a curtain gas interface of 50 psi, ion spray source voltage at 4,500V (negative polarity), gas 1 at 70 psi and gas 2 at 70 psi, source temperature at 450°C, and collision gas at 10 psi. The multiple reaction monitoring (MRM) mode was used to detect and quantify AKG and an internal standard, AKG (1,2,3,4-$^{13}C_4$), with mass-to-charge (*m/z*) transitions at 145 → 101 and 149 → 105, respectively. After generating a calibration curve using the peak area corresponding to different concentrations of the standard, the AKG concentration in each sample was calculated. Samples exhibiting peak areas that exceeded the range of the calibration curve were appropriately diluted. As an internal control, 2 µg/mL AKG (1,2,3,4-$^{13}C_4$), in distilled water was used.

Statistical analysis

All tests were performed in triplicate, and experimental data are expressed as the mean and standard deviation and were evaluated using Student's t-test. A probability value of less than 0.05 was considered statistically significant.

RESULTS

*Isolation and identification of hyaluronidase inhibitor in the supernatant of LAB (***L. paracasei** *NY1301 strain) fermented milk-based beverage*

The hyaluronidase inhibition assay revealed the anti-hyaluronidase effects of the LAB (*L. paracasei* NY1301 strain)-fermented milk-based beverage (Fig. 1). *L. paracasei* NY1301 was selected because of its long history of use in LAB beverages, superior safety compared with other LAB strains, and potential for easy commercialization. The fraction eluted using 25% ethanol exhibited the greatest reduction in enzymatic activity. This fraction was further separated and purified using

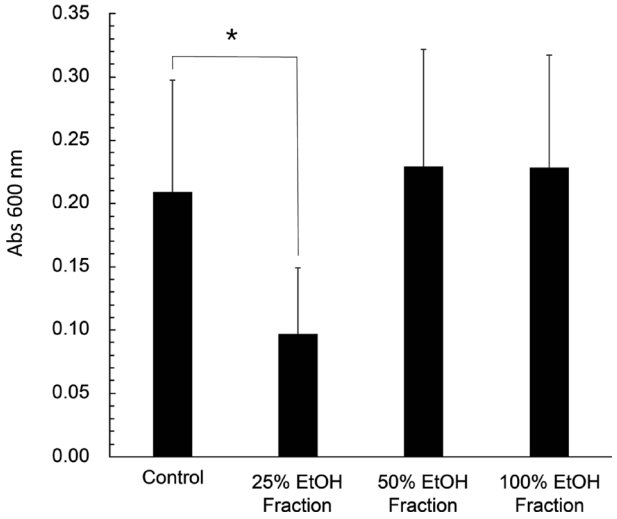

Fig. 1. Hyaluronidase inhibitory activity of lactic acid bacteria (LAB) (*L. paracasei* NY1301 strain)-fermented milk-based beverage-derived fractions separated using ethanol at different concentrations (EtOH fractions) and XAD4. Each bar indicates the mean ± SD for three experiments. *p<0.05 compared with control. Distilled water was used as the control. 25% EtOH Fraction, the fractions eluted with 25% ethanol; 50% EtOH Fraction, the fractions eluted with 50% ethanol; 100% EtOH Fraction, the fractions eluted with 100% ethanol.

an InertSustain C18 column (10 mm × 250 mm, 5 µm), and chromatograms recorded for different fractions (Fr. 1, Fr. 2, and Fr. 3) corresponding to three peaks collected for the hyaluronidase inhibition assay are depicted in Fig. 2A. Figure 2B shows the variable inhibitory effects of the fractions on hyaluronidase activity; the fraction collected at a retention time of approximately 3–5 min (Fr. 2) significantly reduced the enzymatic activity. Therefore, Fr. 2 was purified under modified HPLC conditions, yielding fractions Fr. 2-1, Fr. 2-2, Fr. 2-3, Fr. 2-4, Fr. 2-5, Fr. 2-6, and Fr. 2-7 (Fig. 3A), which exhibited varied inhibitory effects (Fig. 3B). Fr. 2-2, collected at approximately 4–7 min, showed the highest level of anti-hyaluronidase activity; therefore, it was further analyzed using LC/TOF-MS (Figs. 4 and 5). Based on the mass spectral patterns, library searches, and peak waveforms in the UV wavelength range, eight candidate compounds, namely, 2-hydroxyglutarate, uracil, methionine, orotic acid, AKG, nicotinic acid, acetylcarnitine, and cadaverine, were identified. A hyaluronidase inhibition assay using these candidate compounds revealed a significant reduction in the hyaluronidase activity in the presence of AKG (Fig. 6). Therefore, AKG was identified as the inhibitor of hyaluronidase activity in the LAB (*L. paracasei* NY1301 strain)-fermented milk-based beverage. To evaluate the hyaluronidase inhibitory effect of AKG, experiments were conducted to compare the IC_{50} of hyaluronidase with the known

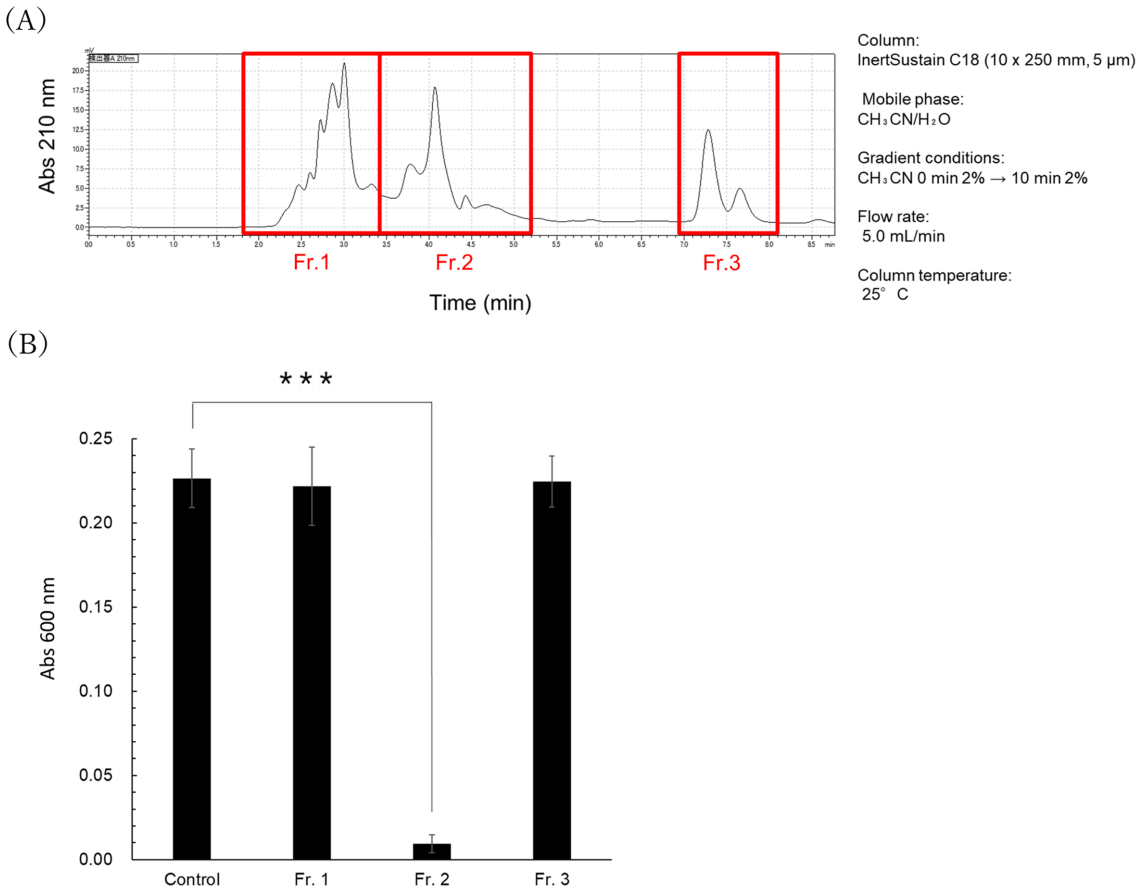

Fig. 2. Characterization of different fractions obtained through the first step separation of hyaluronidase inhibitors using the 25% EtOH fraction and InertSustain C18 (10 × 250 mm, 5 µm). The eluates of the three peaks shown in the figure are Fr. 1, Fr. 2, and Fr. 3, respectively. (A) High-performance liquid chromatography (HPLC) chromatogram corresponding to different fractions. (B) Hyaluronidase inhibitory activity of different fractions. Each bar indicates the mean ± SD for three experiments. ***p<0.001 compared with control. Distilled water was used as the control.

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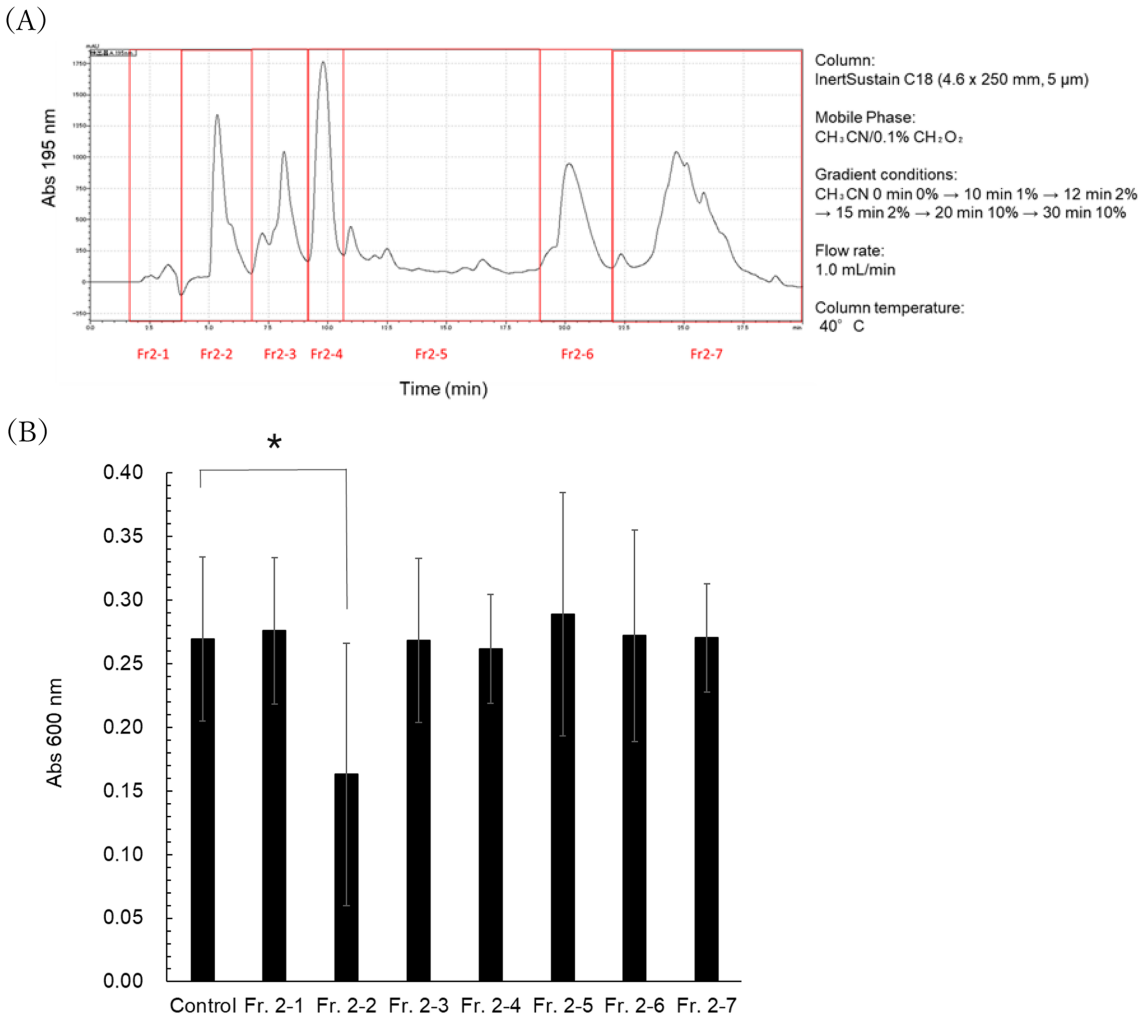

Fig. 3. Characterization of different fractions obtained through the second step separation of hyaluronidase inhibitors using Fr. 2 and InertSustain C18 (4.6 × 250 mm, 5 μm). (A) High-performance liquid chromatography (HPLC) chromatogram corresponding to different fractions. (B) Hyaluronidase inhibitory activity of different fractions. Each bar indicates the mean ± SD for three experiments. *p<0.05 compared with control. Distilled water was used as the control.

strong inhibitor DSCG. As shown in Fig. 7, the IC_{50} of DSCG was 0.36 ± 0.041 mM, whereas that of AKG was 2.82 ± 0.038 mM. The inhibitory effect of AKG was approximately 0.13-fold that of DSCG.

Changes in AKG levels during milk fermentation

During the fermentation process, the supernatant of the LAB (*L. paracasei* NY1301 strain)-fermented milk was collected after 0 and 24 hr of incubation, and the AKG levels were measured in the samples using LC/MS/MS. The AKG levels significantly increased after 24 hr of fermentation compared with that recorded at 0 hr (Fig. 8), indicating that the AKG level was increased by LAB (*L. paracasei* NY1301 strain). However, AKG detected at 0 hr of incubation indicated the presence of AKG in the skim milk.

Screening for high-level AKG-producing LAB

Considering the LAB-induced increase in AKG levels in fermented milk and variable levels of AKG production by different bacterial species and strains, various LAB strains were cultured, and the AKG levels in the culture medium were quantified using LC/MS/MS to select high-level AKG-producing LAB. All LAB species used in the selection were food-grade and formed curds under standard fermented milk production conditions.

The AKG-producing efficacy of seven different strains of LAB is depicted in Fig. 9. Milk samples fermented with *L. acidophilus* JCM1132, *S. thermophilus* NY, and *L. paracasei* JCM1109 exhibited the highest AKG level. Particularly, the results reflected the highest AKG level induced by *L. acidophilus* JCM1132, suggesting that *L. acidophilus* was the most efficient AKG-producing LAB among the test strains.

DISCUSSION

In this study, we evaluated the inhibitory effect of a LAB (*L. paracasei* NY1301 strain)-fermented milk-based beverage on hyaluronidase activity and purified the active component involved in this inhibitory effect using hydrophobic adsorbents and HPLC. Based on the LC/TOF-MS analysis, we identified AKG as an inhibitor of hyaluronidase activity.

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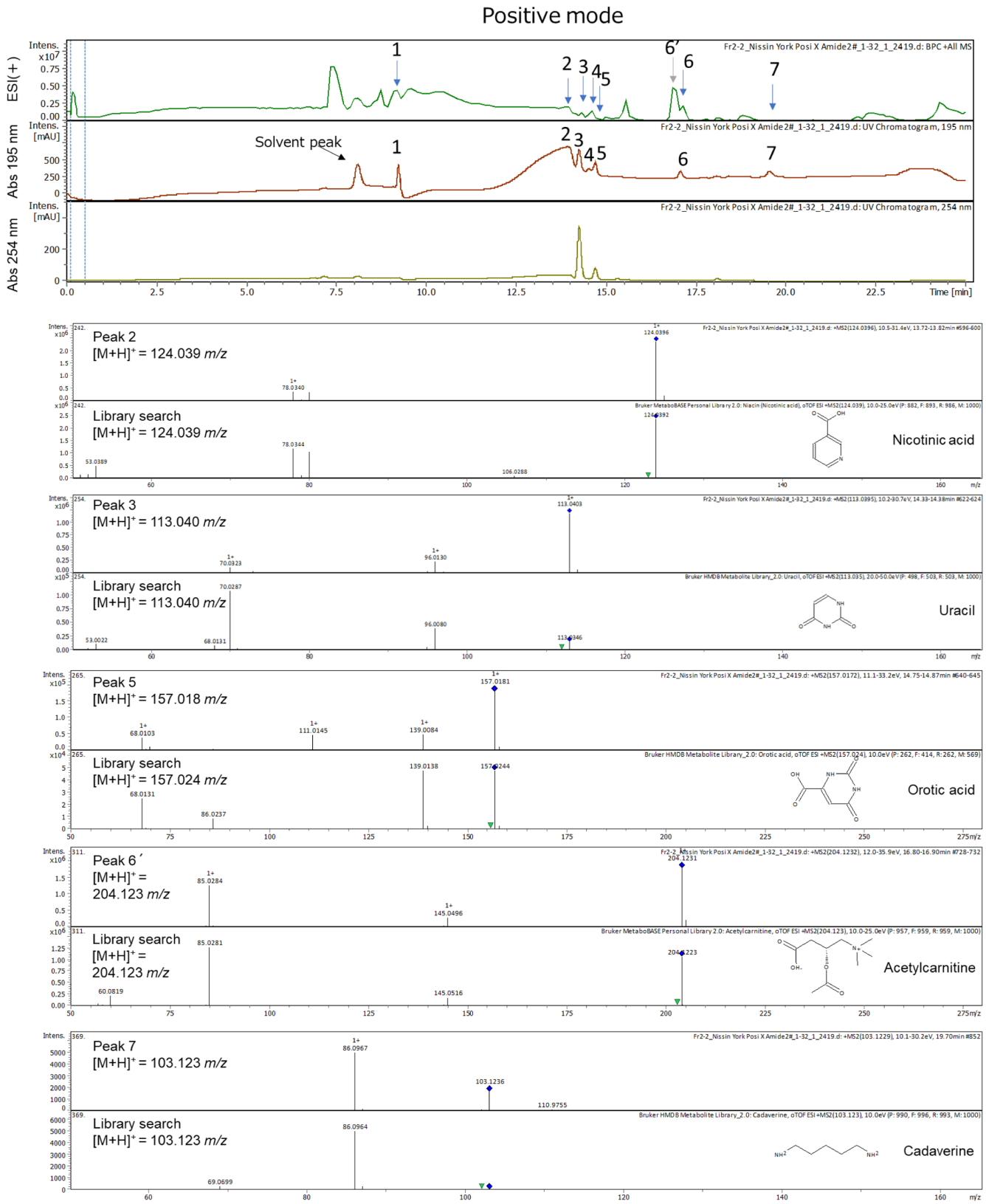

Fig. 4. Separation and analysis of hyaluronidase inhibitors derived from Fr. 2-2 via LC/TOF-MS. Positive mode.
Peak 1, unknown; Peak 2, nicotinic acid; Peak 3, uracil; Peak 4, unknown; Peak 5, orotic acid; Peak 6, acetylcarnitine; Peak 7, cadaverine.

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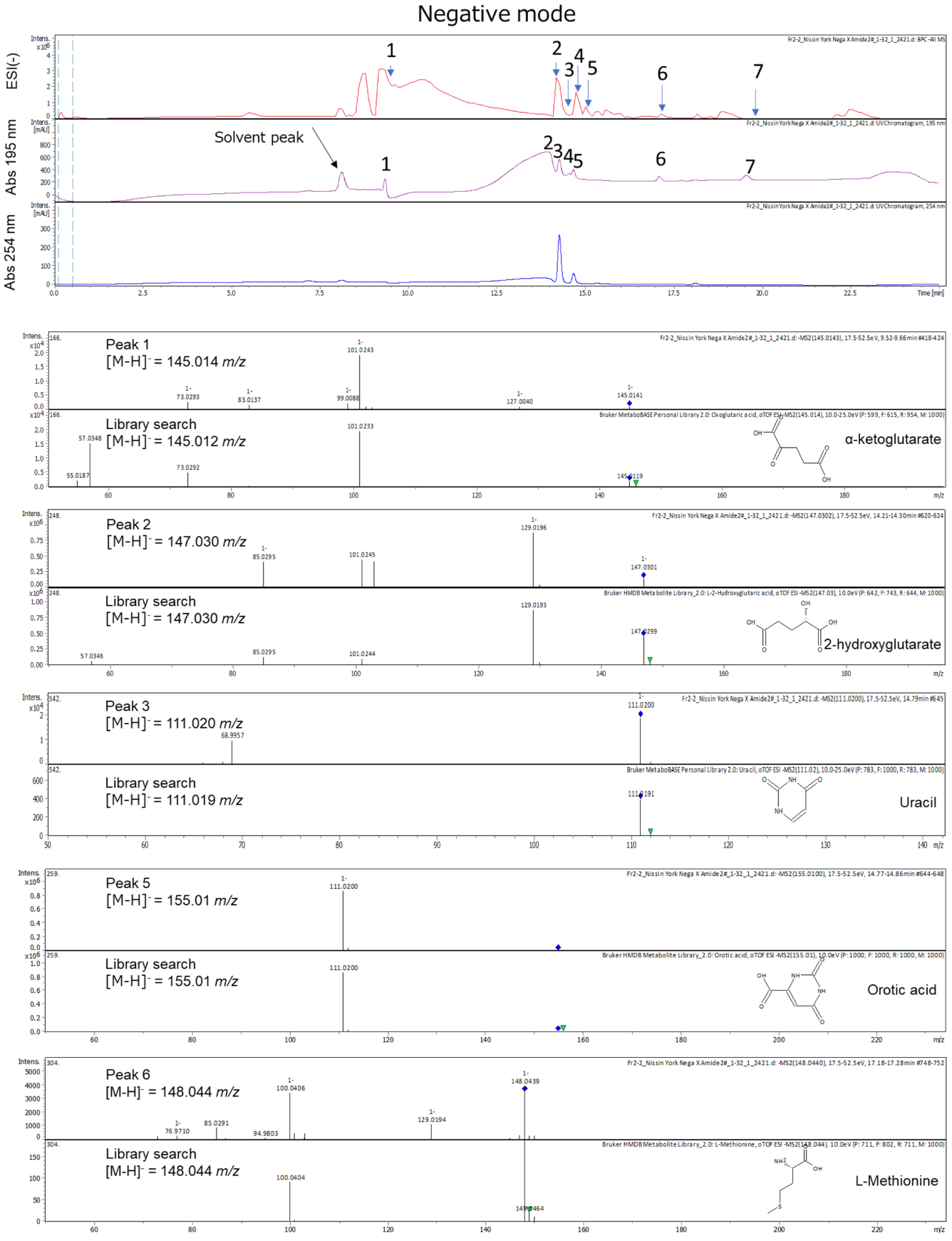

Fig. 5. Separation and analysis of hyaluronidase inhibitors derived from Fr. 2-2 via LC/TOF-MS. Negative mode.

Peak 1, α-ketoglutarate (AKG); Peak 2, 2-hydroxyglutarate; Peak 3, uracil; Peak 4, unknown; Peak 5, orotic acid; Peak 6, L-methionine; Peak 7, unknown.

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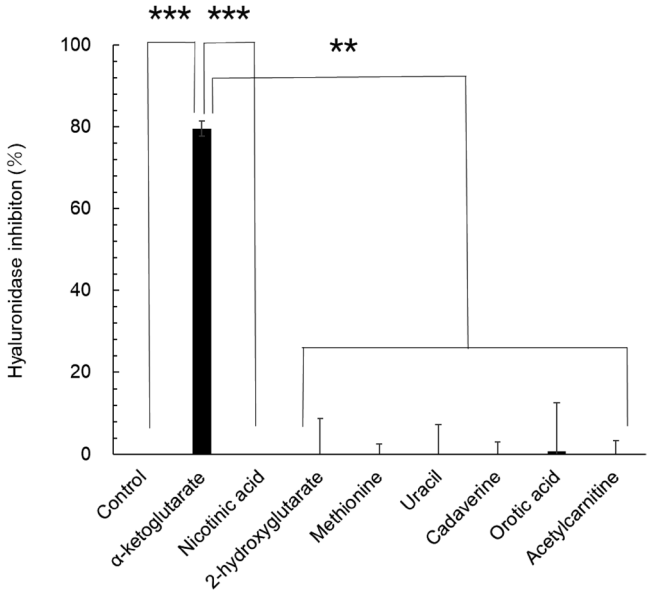

Fig. 6. Inhibition of hyaluronidase activity by compounds predicted through LC/TOF-MS analysis. Each bar shows the mean ± SD for three experiments. **p<0.01 and ***p<0.001 compared with α-ketoglutarate (AKG). The solvents (distilled water or buffer) used to dissolve each candidate compound were used as controls. All compounds were reacted to a final concentration of 4 mM in the reaction solution.

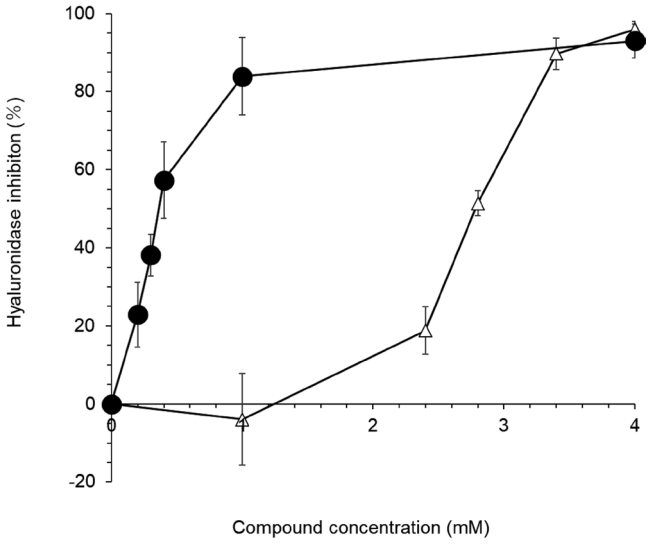

Fig. 7. Inhibitory effects of α-ketoglutarate (AKG) and the strong hyaluronidase inhibitor disodium cromoglycate (DSCG) on hyaluronidase. AKG (unfilled triangles) and DSCG (filled circles) were used to determine the inhibitory effects on hyaluronidase. The IC_{50} indicates the concentration required for 50% inhibition of hyaluronidase and was calculated using the mean of 3–4 measurements. Each bar indicates the mean ± SD for three experiments.

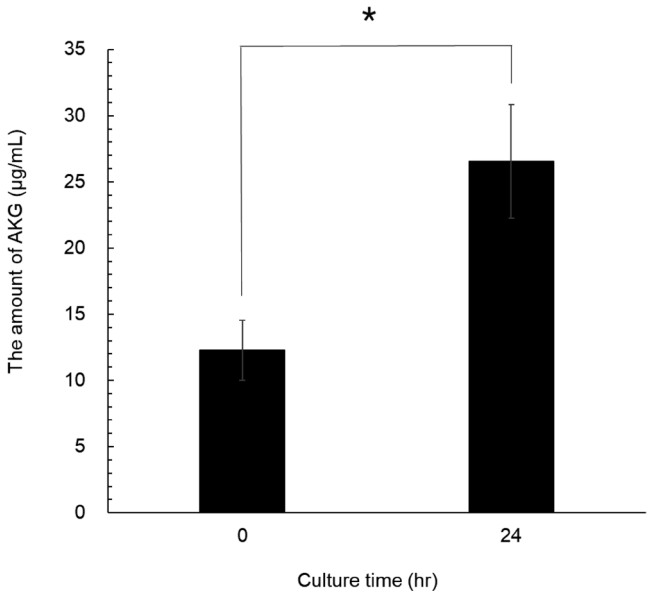

Fig. 8. The change in α-ketoglutarate (AKG) level in lactic acid bacteria (LAB) (*L. paracasei* NY1301 strain)-fermented milk after 24 hr of incubation. Fermented milk samples were collected after 0 and 24 hr of cultivation and centrifuged, the supernatant was collected, and the filtrate was analyzed using LC/MS/MS analysis. Each bar shows the mean ± SD for three experiments. *p<0.05 compared with fermented milk after 0 and 24 hr of cultivation.

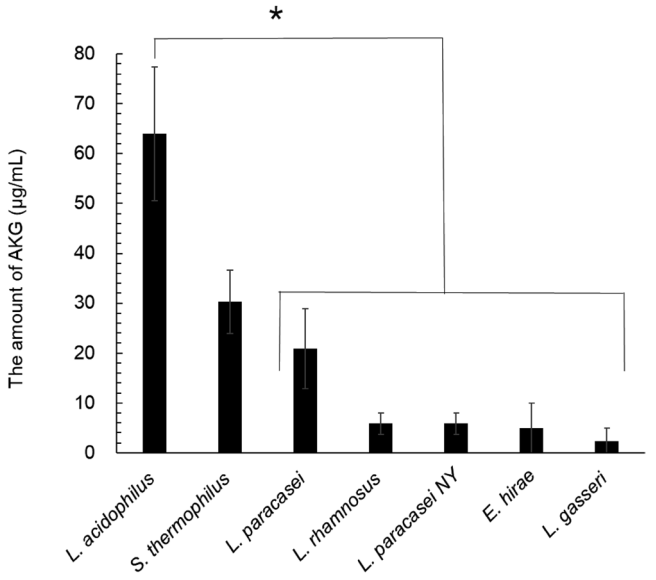

Fig. 9. Variable levels of α-ketoglutarate (AKG) in milk fermented with various lactic acid bacteria (LAB). After culturing the milk media using either *L. acidophilus* JCM1132, *S. thermophilus* NY, *L. paracasei* JCM1109, *L. rhamnosus* ATCC7469, *L. paracasei* NY1301, *E. hirae* ATCC8043, or *L. gasseri* JCM1131 at 37°C for 48 hr, the samples were centrifuged, and the supernatant was collected and filtered through filter paper for LC/MS/MS analysis. Each bar indicates the mean ± SD for three experiments. *p<0.05 compared with culturing the milk media using *L. acidophilus* JCM1132.

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AKG, produced as an intermediate in the tricarboxylic acid (TCA) cycle, is present in various organisms. It contributes to energy production, synthesis of specific amino acids, regulation of redox homeostasis, and detoxification of harmful substances [20]. AKG is naturally present in fermented foods, fruits, and vegetables commonly included in the human diet [21–23]. A previous study revealed that AKG, up to a concentration of 100 μM, exhibits no toxicity on human dermal fibroblasts [24]. These results suggest that the oral intake or topical application of this substance is highly safe.

Recent advances in technology have increased interest in anti-aging research owing to the increased life expectancy of humans. AKG exhibits various antiaging properties, inhibits age-related osteoporosis, and rejuvenates aged mesenchymal stromal/stem cells [25]. Furthermore, it has been reported to extend the lifespans of mice and flies [26, 27]. AKG mediates enhancement in skin hydration and barrier function by regulating the expression of proteins, such as filaggrin, that are involved in the maintenance of epidermal function [28]. It also contributes to the metabolism of skin components, including collagen [24]. However, the present study is the first to demonstrate the inhibition of hyaluronidase activity by AKG.

We investigated the LAB (*L. paracasei* NY1301 strain)-mediated increase in AKG levels in fermented milk. The increase in AKG level during the fermentation of the milk sample validated that AKG was produced by the LAB under the culture conditions. Previous studies on the metabolism of AKG by LAB have shown that some lactobacilli lack a part of the TCA cycle and cannot metabolize citric acid to form AKG [29] and that glutamate can be converted to AKG by glutamate dehydrogenase (GDH) [30, 31]. Screening for high-level AKG-producing LAB revealed the high efficacy of *S. thermophilus* and *L. acidophilus* to produce AKG. GDH from these LAB has been reported to be more active than that from other LAB strains [30, 32], suggesting that GDH may be involved in the production of AKG in this strain. Therefore, we measured AKG production in a glutamate-supplemented medium under the same conditions for selecting high AKG-producing LAB. We observed no increase in AKG production by *S. thermophilus* NY (data not shown). These results suggest the involvement of mechanisms other than GDH, such as amino acid aminotransferases that consume AKG as a substrate. GDH is also indirectly involved in the catabolic metabolism of other amino acids. Lactobacilli possessing GDH activity were able to degrade amino acids more effectively when glutamate was available [33]. This mechanism may be attributed to an aminotransferase reaction using AKG and amino acids. Therefore, to increase the total amount of AKG, it may be necessary to consider the balance between the activities of GDH and aminotransferases responsible for AKG biosynthesis and degradation. However, the detailed mechanism of AKG remains unclear and requires further exploration.

Our study has some limitations, and more research is needed to clarify the relationship between LAB and hyaluronidase inhibitors. Although we showed that LAB strains have excellent AKG-production capacity under certain conditions, this result may vary depending on the temperature and media. The optimum temperature and medium conditions differ among LAB strains and are related to the amount of bacteria and metabolites. Although the assay conducted was intended for application to fermented milk products, which was the purpose of this study, the intended application does not seem to have a major impact. Thus, to comprehensively understand the underlying mechanism, considering the culture conditions for each strain will be necessary.

In conclusion, we, for the first time, identified LAB-derived AKG as an inhibitor of hyaluronidase activity. Among various hyaluronidase inhibitors, AKG stands out as a particularly safe compound, as it is naturally present in various foods and has been consumed for a long period of time. Furthermore, screening for high-level AKG-producing LAB emphasized that several LAB strains can be used to produce milk with anti-hyaluronidase properties. Specifically, *L. acidophilus* JCM1132 and *S. thermophilus* NY are potential high-level AKG-producing LAB strains. The findings of this study could help better understand the relationship between skin health and LAB and develop safe functional foods and cosmetics enriched with AKG.

CONFLICTS OF INTEREST

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