Evaluation of the Functional Properties of Pork Meat Fermented with Psychrotrophic Lactic Acid Bacteria

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Abstract— **The food functionality of model pork sausages fermented with psychrotrophic lactic acid bacteria was evaluated by analyzing protein degradation, the inhibitory activity against angiotensin I-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP IV), as well as the effect on growth promoting activity in MC3T3-E1, an osteoblast-like cell. The peptide content of fermented model sausages was significantly higher than that of non-fermented sausages ($p < 0.05$). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis indicated that the staining intensity of the low-molecular-weight bands (<30 kDa) of water-soluble proteins obtained from fermented model sausages was slightly higher than that of proteins obtained from non-fermented sausages. This suggests that lactic acid fermentation can successfully degrade muscle protein even at low temperatures. Inhibitory effects of the water-soluble protein fractions obtained from model sausages on ACE and DPP IV were assessed using *in vitro* assays. Fermented model sausages displayed higher ACE- and DPP IV-inhibiting activity than non-fermented sausages ($p < 0.05$). Furthermore, alkaline phosphatase activity and collagen synthesis, widely recognized as biochemical markers for osteoblastic activity, were significantly increased in fermented model sausages compared with non-fermented sausages ($p < 0.05$). These results suggest that lactic fermentation at cold temperatures can improve the food functionality of meat products.**

Keywords— **Cold fermentation, Muscle protein degradation, Physiological function**

I. INTRODUCTION

In recent years, much attention has been paid to safe and healthy food, including milk-based fermented animal products such as yogurt and cheese. However, meat and meat products are not considered good for health, despite their high protein content and excellent nutritional value, because meat is rich in fat and cholesterol [1].

We have performed various quality evaluations of processing methods, such as lactic acid fermentation and retort sterilization, with the aim of developing meat products having greater added value. After lactic fermentation, the colour and physical properties of meat are markedly improved [2]. This process is carried out at the optimal temperature (30–37°C) for most bacterial growth, and data have been obtained demonstrating improvements in functionality, such as the generation of peptides with inhibitory activity against angiotensin I-converting enzyme (ACE), which may counteract blood pressure elevation [3]. The optimum temperature for most lactic acid bacteria (LAB) is around 30°C, but bacteria that cause food-borne diseases will also thrive at this temperature. In recent years, we have tried to apply cold fermentation to improve the quality of meat products without compromising safety by using psychrotrophic LAB, nearly identified as *Lactobacillus sakei* [4]. We observed that myofibril fragmentation and muscle protein degradation in cured meat is increased during fermentation with psychrotrophic LAB, even at cold temperatures [5].

Research on bioactive peptides originating from food proteins has been recently reported [6]. Therefore, it is reasonable to expect that functional low-molecular-weight peptides can be formed as a result of protein decomposition that occurs even during cold fermentation. Although some previous studies have demonstrated that lactic acid starter culture and subsequent fermentation could improve the safety and quality of meat products [7,8], very few reports have focused on the food functionality of meat fermented with psychrotrophic LAB. In addition, many factors with ACE inhibitory activity have been obtained from meat and meat products so far [3,6], but other physiological functions in meat have not been well studied. Interestingly, it was recently reported that ACE inhibition might improve the bone metabolism directly or indirectly [9].

In the present study, we evaluated the food functionality of model pork sausages fermented with

psychrotrophic LAB at 4°C. As indices of physiological activity, we focused on the inhibitory activity against ACE, and dipeptidyl peptidase IV (DPP IV), which plays a major role in glucose metabolism, and on the growth-promoting activity in osteoblast-like cell cultures.

II. MATERIALS AND METHODS

A. Sample preparation

Fresh commercial pork (ham) was purchased from a local wholesaler. Visible fat and connective tissue were carefully removed, and the pork was cut into small cubes (approximately 3 cm in diameter). The meat was cured using the following agents: 2.0% sodium chloride, 0.02% sodium nitrite, 0.05% potassium nitrate, 0.3% sodium tripolyphosphate, 0.05% sodium ascorbate and 2.0% glucose.

Cured meat added with 5% crushed ice was immediately cut for 60 s using a bowl cutter. Then, the cured meat emulsion was divided into four portions. Each three of them was inoculated with *Lactobacillus sakei* D-1001 (*Lb*. D-1001) and 2 different strains (*Lb*. No.3 and *Lb*. No. 12, nearly identified as *Lb*. sakei) out of 16 psychrotrophic LAB strains that we possess [4] at 10^6 log CFU/g meat, respectively. The inoculated samples were fermented at 4°C. After 3 days of fermentation, the meat emulsions (100 ± 5 g for each sausage) were stuffed into a polyvinylidene chloride casing (40 mm in diameter) and further fermented at 4°C for 2 days. As a control, the remaining non-inoculated portion of the cured meat emulsion was stored at 4°C for 3 days and was stuffed into the casings as described above. Chronological sampling during fermentation was carried out for measuring the pH of the cured and fermented meat, and for viable counts of LAB.

Then, the raw fermented and non-fermented samples were cooked at 75°C, holding for 30 minutes after the meat reached an internal temperature of 63°C. After cooking, all the model sausage samples were cooled under ice water for 30 min.

Both fermented and non-fermented samples were defatted with diethyl ether, homogenized in a 6-fold volume of distilled water, and centrifuged (35,000 ×*g*, 15 min, 4°C). The resulting supernatants were used as water-soluble protein fractions.

The protein concentration of each fraction was determined by the biuret method (at 550 nm).

B. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and peptide content

SDS-PAGE: The protein distribution in the water-soluble protein fractions from the model sausages was analysed by SDS-PAGE on a gradient slab gel (7.5–20%). The gels were stained with Coomassie Brilliant Blue R-250.

Peptide content: Fractions soluble in 2% trichloroacetic acid (TCA) were prepared by adding an equal volume of 4% TCA to the water-soluble fraction. The resulting 2% TCA-soluble fractions were analysed for their peptide content according to the method described by Mikami et al. [10].

C. ACE and DPP IV inhibitory activity

The water-soluble protein fractions obtained from the model sausages were dialyzed in distilled water using a dialysis membrane (molecular weight cutoff: < 500 Da) and then lyophilized. The lyophilized material was dissolved in distilled water, filtered through a syringe filter (0.22 μm) and used as the sample for the ACE and DPP IV inhibitory activity assay.

ACE inhibitory activity was measured according to the method of Cushman and Cheung [11]. In this method, ACE inhibitory activity is quantified by the reaction of hippuryl-histidyl-leucine with ACE to form hippuric acid, the absorbance of which is measured at 228 nm.

DPP IV inhibitory activity was measured according to the method described by Ikehara et al. [12]. DPP IV inhibitory activity was quantified by the reaction of Gly-Pro-*p*-nitroanilide tosylate with DPP IV to release *p*-nitroaniline.

The decrease in absorbance is proportional to the amount of inhibition of ACE or DPP IV caused by substances in the sample. The peptide concentration (mg/ml) required to inhibit 50% of ACE or DPP IV activity was defined as the IC_{50} value.

D. Growth promotion of osteoblast cells

MC3T3-E1 cells (RCB1126) were cultured at 37°C in a 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM). Unless otherwise specified, the medium contained 10% foetal bovine

serum (FBS).

Alkaline phosphatase (ALP) activity and collagen content were determined according to the method described by Choi [13], with slight modifications. In brief, after the cells were cultured at a density of 2.5 × 10^5 cells/ml in a culture dish for 3 days, the medium was replaced with FBS-free α-MEM; the cells were cultured in this medium in the presence or absence of the water-soluble protein fractions (0.5 mg/ml) for 3 days.

ALP activity: After incubation, the medium was removed and the cell monolayer was gently washed 3 times with phosphate buffered saline (PBS). The cells were lysed by sonication in a lysis solution (0.25 M sucrose), and the cell lysates were centrifuged at 2000 ×*g* for 3 min. The resulting supernatants were used for measurement of ALP activity and protein concentration. ALP activity and protein concentration were measured using an ALP Assay Kit (Wako) and a BCA Protein Assay Kit (Pierce), respectively. ALP activity was calculated according to the manufacturer's protocol and expressed as nmol of p-nitrophenyl phosphate per min per μg of cellular protein.

Collagen content: After 3 days of incubation in the presence or absence of the water-soluble protein fractions, the collagen content was measured using the Sircol Collagen Assay Kit (Biocolor). This assay uses a quantitative dye-binding method designed for the analysis of collagens from mammalian cell cultures. The collagen content was calculated according to manufacturer's protocol and data were expressed as mg of collagen per ml of culture supernatant.

III. RESULTS AND DISCUSSION

Changes in water-soluble protein distribution because of lactic acid fermentation at low temperature were analysed by SDS-PAGE (Fig. 1). The staining intensity of the 40–45 kDa bands of *Lb*. D-1001, *Lb*. No. 3 and *Lb*. No. 12 inoculated sausages markedly decreased because of cold fermentation. Conversely, the staining intensity of low-molecular-weight bands (< about 30 kDa) of fermented samples was slightly higher than that of non-fermented samples, especially for *Lb*. No. 3 and 12 inoculated samples. Furthermore,

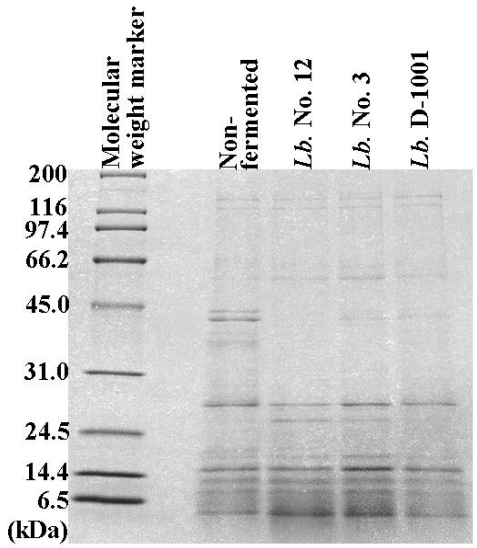

Fig. 1 SDS-PAGE analysis of water-soluble proteins prepared from non-fermented and fermented model sausages using psychrotrophic LAB.

the peptide content in the water-soluble protein fractions from all fermented sausages was significantly higher than that of the control (data not shown). We have previously reported that myofibril fragmentation and myofibrillar protein degradation of pork meat are markedly accelerated by fermentation with psychrotrophic LAB [5]. In the present study, viable counts of LAB in all inoculated samples were maintained at 10^7–10^8 CFU/g meat, and pH values decreased to approximately 5.3 during cold fermentation (data not shown). These data suggest that fermentation by psychrotrophic LAB results in low pH and degradation of muscle protein, including water-soluble proteins, under a low-temperature condition.

Various bioactive peptides including those of animal food origin have been recently discovered [3,6], and we have found a novel pentapeptide (EQHLG) having ACE inhibitory activity that was isolated from a myosin solution fermented with *Lactococcus lactis* subsp. *lacis* IFO (NBRC)-12007 at 30°C. Therefore, we first examined whether the ACE and DPP IV inhibitory activities of meat were affected by cold fermentation. In tests to determine the extent of ACE and DPP IV inhibition, the IC_{50} values of all fermented samples were significantly lower than those of control (data not shown). Thus, the fermented sausages showed higher inhibitory activities for these enzymes than the control.

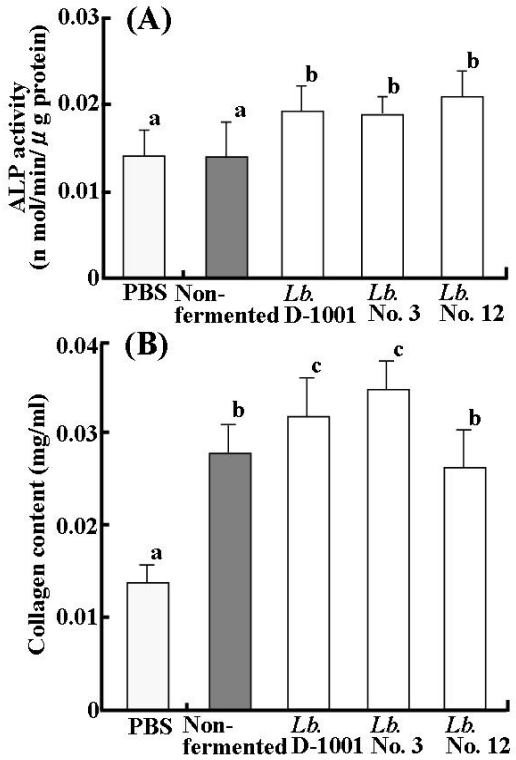

Fig. 2 Effects of water-soluble protein fractions obtained from non-fermented and fermented model pork sausages on ALP activity (A) and collagen synthesis (B) in MC3T3-E1 cells.

Data represent the mean ± SD of at least four independent experiments. Values with different superscript letters are significantly different ($p < 0.05$).

We next examined the effect of the fermented and non-fermented sausages on the growth of a culture of osteoblast-like cells (MC3T3-E1). Figures 3A and B show the effects of the water-soluble fractions obtained from the model sausages (0.5 mg/ml protein) on ALP activity and collagen content in MC3T3-E1 cells, respectively. As shown in Fig. 3A, the ALP activity was significantly increased in all fermented sausages compared with that in the non-fermented control sausage and PBS (used as vehicle control) ($p < 0.05$). In addition, the collagen content significantly increased in the fermented sausages as compared to the non-fermented control sausage, except in the *Lb*. No. 12 inoculated sausage.

Therefore, cold fermentation with our *Lb.sakei* strains may be useful not only for food safety but also for improving the functionality of meat products.

IV. CONCLUSIONS

This study indicated that cold fermentation with psychrotrophic LAB could improve the various food functionalities of meat products.

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