Buffering Effects of Calcium Salts in *Kimchi*: Lowering Acidity, Elevating Lactic Acid Bacterial Population and Dextranucrase Activity

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This study investigates the buffering effects of calcium salts in *kimchi* on the total acidity, microbial population, and dextranucrase activity. Calcium chloride or calcium carbonate was added to *dongchimi-kimchi*, a watery radish *kimchi*, and the effects on various biochemical attributes were analyzed. The addition of 0.1% calcium chloride produced a milder decrease in the pH after 24 days of incubation, which allowed the lactic acid bacteria to survive longer than in the control. In particular, the heterofermentative *Leuconostoc* genus population was 10-fold higher than that in the control. When sucrose and maltose were also added along with the calcium salts, the dextranucrase activity in the *kimchi* was elevated and a higher concentration of isomaltooligosaccharides was synthesized when compared with the control. Calcium chloride was determined as a better activator compound of dextranucrase than calcium carbonate, probably because of its higher solubility. Therefore, the results of this study confirm the ability of the proposed approach to modulate the *kimchi* fermentation process and possibly enhance the quality of *kimchi* based on the addition of dietary calcium salts.

**Keywords:** Dextranucrase, *kimchi*, *Leuconostoc*, calcium carbonate, calcium chloride, isomaltooligosaccharide

*Leuconostoc* species are heterofermentative lactic acid bacteria (LAB) and important bacterial populations in *kimchi* or sauerkraut from the initial to the middle stages of fermentation [7, 14]. During these stages, these bacteria produce various constituents, such as lactic acid, acetic acid, alcohol, CO₂, and mannitol, all of which contribute to the flavor of the fermented foods. For this reason, in 2005, a leading *kimchi* producer in Korea began to use *L. mesenteroides* as a starter culture for taste improvement and quality control. However, the microorganisms belonging to the *Leuconostoc* genus are more acid-labile than other LAB and their growth is inhibited by the lactic acid excreted during *kimchi* fermentation below pH 4.0 [15]. In addition, the acid content influences the taste of *kimchi*; a total acidity of 0.6–0.8% is recognized to give the best taste, whereas acidity above this range produces a strong acid taste and lowers the quality of the *kimchi* [12]. The concentration of lactic acid in *kimchi* can be reduced by the addition of calcium salts to neutralize the lactate into calcium salt (*i.e.*, calcium lactate).

The dextranucrase (E.C. 2.4.1.5) secreted by *Leuconostoc* species transfers the glucose moiety of sucrose to form dextran-like glucan, and catalyzes the transfer of glucose from sucrose (donor) to other sugars (acceptors) by linking an α-(1→6)-glucosyl bond [20, 22]. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor products is usually produced, and maltose has been shown to be the best acceptor molecule in experiments using *L. mesenteroides* NRRL B-512F [2]. Therefore, using this reaction, the current authors already proposed a symbiotic oligosaccharide synthesis method for *kimchi* and fermented milk [5, 6, 8]. In the *kimchi* manufacturing process, the simple addition of sucrose and maltose to the ingredients has achieved a high conversion yield of isomaltooligosaccharides (IMOs) via the reaction of the dextranucrase excreted by the inherent *Leuconostoc* bacteria.
Miller and Robyt [16] also reported that calcium ions are an activator of dextranase based on increasing the V_max and decreasing the K_m for sucrose.

Accordingly, this study used the addition of calcium salt to kimchi for two goals: first, to decrease the lactic acid content through the formation of calcium salts to reduce the acid stress against leuconostocs and thereby maintain the population of beneficial microflora; and second, to activate the dextranase excreted from leuconostocs to synthesize more IMOs in kimchi. For this purpose, calcium chloride and calcium carbonate were selected as the calcium salts, since they are soluble in water and acceptable for dietary use. After adding the salts to the kimchi preparation, the microbial cell counts, including the LAB and leuconostocs, were monitored, and the dextranase activities with the amounts of IMOs synthesized were assayed during the whole period of kimchi fermentation.

**Materials and Methods**

**Materials**
The dextranase, sucrose, and standard chemicals were all purchased from Sigma Inc. (St. Louis, MO, U.S.A.), and the maltose was from Duksan Pharmaceuticals (Yongin, Korea). The lactobacilli MRS broth was from Difco (Detroit, MI, U.S.A.), and cubic plastic jars with sealing lids were used as the fermentation vessel. The radishes, red peppers, green onions, and other constituents were purchased from a local grocery store.

**Bacterial Strain and Enzyme**
The dextranase was obtained using *Leuconostoc citreum* KACC 91035, which is a psychrotrophic strain secreting highly active dextranase across a broad temperature range [6]. The strain was cultivated in a sucrose broth (500 ml) under aerobic conditions for 48 h at 28°C. The S-medium was composed of 24.7 g sucrose, 4.2 g peptone, 4.2 g yeast extract, 20 g K_HPO_4, 0.2 g MgSO_4·7H_2O, 0.1 g NaCl, 0.1 g FeSO_4·7H_2O, 0.1 g MnSO_4·H_2O, and 0.13 g CaCl_2·2H_2O per liter of distilled water [11], where 0.5–1 mg/ml Tween 80 was added for enzyme stabilization. To determine the effect of the calcium ions on the dextranase, the enzyme was purified using the method described by Miller et al. [15, 16]. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 10 min at 4°C. To digest the dextran, lyophilized dextranase was added to the culture supernatant, which was then dialyzed overnight against 0.2 M sodium acetate (pH 5.2), 0.05 M NaCl in regenerated cellulose dialysis membrane tubing (3.5 kDa molecular mass cutoff; Spectra/ Por, Spectrum Laboratories Inc., CA, U.S.A.) at 4°C with two changes of buffer. The dialyzed solution was concentrated by dehydration with polyethylene glycol 6000 at 4°C overnight. The dextranase was further purified by DEAE-cellulose column chromatography equilibrated with 0.02 M sodium acetate (pH 5.2) and 0.05 M NaCl with a 0.2 M NaCl linear gradient in 0.02 M imidazole-HCl (pH 6.7). The purified enzyme fractions were then filtered through a 0.22-μm filter and stored at -70°C until use. The dextranase activities were measured by assaying the changes in the fructose concentration [19] after modification [21] using dinitrosalicilic acid (DNS) methods [17] in a 20 mM Na-acetate buffer solution (pH 5.2) containing 100 mM sucrose, 1 mM CaCl_2, and 0.02% NaN_3. One unit of dextranase was defined as the amount of enzyme used to produce 1 μmol of fructose per minute at 25°C.

**Kimchi Preparation and Fermentation**

* Dongchimi-kimchi is a popular wetary radish *kimchi*. The whole radish (800 g) was washed, the outer layer peeled off and cut into small pieces. The pieces were then mixed with salt (40 g) in a plastic jar and incubated at 20°C for 6 h until they became soft. Next, the salted radish and extract solution were mixed with crushed garlic (10 g), ginger (3 g), and chopped green onions (20 g). To synthesis IMOs, sucrose and maltose were added to make a final concentration of 1% (w/v). The jar was then filled with 41 of drinking water and tightly sealed with a plastic lid. [3]. The *dongchimi* samples with added sucrose and maltose [each 1% (v/v)] were grouped as follows: A, control without calcium salt; B, *dongchimi* with 0.1% calcium chloride; and C, *dongchimi* with 0.1% calcium carbonate. The fermentation temperature was kept at 20°C for 2 days after reaching the maximum level of *Leuconostoc* sp. growth and dextranase activity. Thereafter, the temperature was dropped to 4°C to reduce the bacterial growth and sugar consumption. *Kimchi* samples (10 ml liquid) were harvested periodically to analyze the microbiological and physicochemical changes during fermentation.

**Microbiological Analysis**
The viable bacteria were counted using MRS and phenylethanol agars (Difco, U.S.A.) with 2% sucrose (PES [15]). Each sample was serially diluted with 0.85% physiological saline. The total number of LAB was determined by spread-plating onto the MRS agar and incubating at 28°C for 48 h [10], and the *Leuconostoc* genus population was counted by spread-plating onto the PES agar after incubation at 20°C for 48 h [13].

**Chemical Analysis**
The pH of the *dongchimi* samples was measured using a pH meter (IQ 240, IQ, Scientific Inc., U.S.A.) and the titratable acidity was determined by titrating with 0.1 N NaOH to an end point of pH 8.3 [1]. The percentage of lactic acid in the sample was calculated by multiplying the volume of the NaOH solution (ml). For a quantitative analysis of the sugars, 1 ml of each sample was loaded onto a Merck K5 TLC plate and developed three times with acetonitrile/distilled water [85:15 (v/v)]. The separated sugars were then detected by dipping the plate in ethanol containing 0.5% (w/v) α-naphthol and 5% (v/v) sulfuric acid, followed by heating at 110°C for 5 min. The final sugar analysis was performed using the Sigmagel program (Sigma Inc., U.S.A.) [6].

**Results and Discussion**

**Purification of Dextranase**

To investigate the effects of calcium salts on dextranase activity, *L. citreum*, as a representative species of the *Leuconostoc* genus, was cultured in an S-medium and the production of dextranase was induced by the addition of sucrose. The dextran polymer produced by dextranase
when using sucrose as the glucosyl donor usually has an adverse effect on the enzyme purification process owing to the formation of a dextran–enzyme complex. Therefore, dextranase (9 mg, 0.09 IU/mg) was added to break down the polymer selectively and the culture was dialyzed for 24 h. The crude enzyme mixture was then analyzed by DEAE-cellulose column chromatography (Fig. 1) and the fractions (10 and 11) retaining enzyme activity were pooled and concentrated.

**Effects of Calcium Salts on Dextransucrase Activity**

To determine the effects of calcium salts on dextransucrase, calcium carbonate (CaCO$_3$) and calcium chloride (CaCl$_2$) were used. The salt concentrations used were 0.1% or 0.5% and two different solutions were used for the enzyme reaction; a standard buffer solution (20 mM Na-acetate) for the dextransucrase reaction, and dongchimi-kimchi liquid to examine the real effect of calcium salts in kimchi. As shown in Fig. 2, the dextransucrase activities increased in both solutions with the addition of CaCl$_2$ or CaCO$_3$, and CaCl$_2$ had a higher activation effect than CaCO$_3$ in both cases. In the sodium acetate buffer, the addition of 0.1% and 0.5% CaCl$_2$ increased the dextransucrase activity by 10% and 60%, respectively, when compared with the control, whereas the addition of CaCO$_3$ increased the dextransucrase activity by 5% and 10%, respectively. When the dextransucrase activities were measured in the real *kimchi* solution, the addition of the calcium salts produced the same results as those obtained with the sodium acetate buffer solution (Fig. 2).

**Effects of Calcium Salts on LAB Population**

After adding the calcium salts [0.1% (w/v)], the *dongchimi kimchi* was fermented at 20°C for 2 days, then stored at 4°C, and the biochemical changes of the *kimchi* were monitored. As shown in Fig. 3, the initial pH of sample B containing CaCl$_2$ and sample C containing CaCO$_3$ was 7.2 and 6.0, respectively, which was higher than that of the control A (pH 5.2). During the 24-day fermentation period, the pH of the control, sample B, and sample C decreased to 4.1, 5.2, and 4.4, respectively, indicating that the calcium salts had a neutralizing effect in the acid solution of the *kimchi* from the beginning to the end of the fermentation. Moreover, whereas the total acidity of samples gradually increased during the fermentation period, the rate of increase for the samples with the calcium salts (B and C) was lower than that for the control (A). In particular, sample B with CaCl$_2$ had a lower total acidity value (0.15%) than the control (0.4%) and sample C (0.25%), which was probably because calcium chloride is more easily dissociated than calcium carbonate which is less soluble in water. When the same experiment was conducted with a 0.5% addition of calcium salts, the salts were not dissolved completely and made the *kimchi* turbid.

Fig. 4 shows the changes in the viable counts of LAB and the *Leuconostoc* genus during the spontaneous fermentation of the *dongchimi-kimchi* after the addition of the two calcium salts. The total LAB and leuconostoc counts for the three groups (initial counts; 10$^3$ CFU/ml) increased at a similar rate for about 7 days, and then decreased until the end of the storage period (24 days). *Leuconostoc* spp. were observed as one of the major isolates among the LAB in the *kimchi* samples. Although no significant changes in the viable bacterial counts were observed between the three groups, the total LAB counts for sample C with 0.1% CaCO$_3$ were much higher (about

**Fig. 1.** Dextransucrase purification chromatogram obtained from DEAE-cellulose column chromatography. Curve absorbance of protein concentration measured at 280 nm; line, concentration of NaCl solution; A, dextransucrase fractions.

**Fig. 2.** Comparison of dextransucrase activity in buffer with sodium acetate and acetic acid; pH 5.2 (A) and *dongchimi-kimchi* juice (B) with addition of calcium salts. The control indicates the dextransucrase activity without calcium salts.
10–100 fold) than those for the other two samples (A and B). Furthermore, sample C also exhibited the highest Leuconostoc counts until the end of the fermentation period.

Therefore, these results confirmed that calcium chloride, a well-known neutralizing agent [24, 25], extended the survival of LAB, including leuconostocs, under acidic kimchi conditions.

Effects of Calcium Salts on IMO Production

The glucosyl transfer reaction of dextranucrase is used for the synthesis of IMOs in kimchi. In previous work using the dextranucrase from L. mesenteroides NRRL B-512F, an equimolar addition of sucrose and maltose provided the best conditions for a higher production of panose, the major component of IMOs [20]. Thus, 1% sucrose and 1% maltose were added to the kimchi with calcium salts during the preparation, and the changes in the sugar concentration in the kimchi during the fermentation were then analyzed (Fig. 5). As expected, an acceptor reaction of dextranucrase occurred, where the glucose residue was transferred from sucrose to maltose, resulting in panose as the major product of the acceptor reaction [8]. In all cases, the sucrose (1%) was rapidly consumed within 2 days, whereas only about half of the maltose (0.5%) was used as acceptor molecules. The oligosaccharide concentrations were highest after 4–7 days and these levels were maintained for the remainder of the fermentation at 4°C without any remarkable decomposition. When the concentration of panose was compared among the samples, samples A, B, and C reached their maximum level of 0.50% (on the 14th day), 0.70% (10th), and 0.65% (10th), respectively, and the two samples including the calcium salts maintained a higher concentration of panose throughout the fermentation.
Therefore, the experimental results indicated that the calcium salts affected the *kimchi* in two ways; first, the calcium salts protected the LAB from acids by neutralization, allowing the LAB population to increase; and second, the leuconostocs were able to increase the amount of dextransucrase owing to the less harsh conditions after neutralization, while the calcium salts activated dextransucrase activity to facilitate the production of more IMOs from sucrose and maltose. Similarly, it has also been reported that calcium carbonate and calcium chloride in the growth medium increase the production of other enzymes, such as amylase [23] and fructosyltransferase [25].

Another interesting aspect of the experiments was the synthesis of calcium lactate. The addition of calcium salts to *kimchi* is known to result in the synthesis of calcium lactate, based on the combination of calcium ions and two lactic acids [4, 18]. As a result of this reaction, the total acidity levels and pH of the *kimchi* did not change significantly in samples B and C (Fig. 2). Calcium lactate is currently used as a representative calcium-fortifying supplement for osteoporosis patients, owing to its higher bioavailability to be absorbed in the intestine rather than free calcium ions [2, 9]. Although assaying the calcium lactate in *kimchi* liquid that contains both free calcium ions and lactic acid is not technically feasible at this moment, it is nonetheless reasonable that the calcium lactate synthesized may provide additional health benefits to the *kimchi* manufacturing process in addition to the increased LAB and synthesis of IMOs.

In conclusion, the proposed method of adding calcium salts during *kimchi* preparation was shown to enable the prolonged growth of LAB and overproduction of beneficial oligosaccharides in *kimchi* by the activation or overproduction of dextranase. Therefore, the application of this method will permit the development of new function-added lactate foods.

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**References**


