Cinnamaldehyde Inhibits Enzymatic Browning of Cut Lettuce by Repressing the Induction of Phenylalanine Ammonia-Lyase without Promotion of Microbial Growth

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ABSTRACT: Cinnamaldehyde treatment inhibited the browning of cut lettuce during cold storage. In this study, to clarify the mechanism of inhibitory action of cinnamaldehyde against the browning and to show its microbiological merit, its effect on the browning of cut lettuce was compared to that of mild heat treatment. Both cinnamaldehyde and mild heat treatments inhibited the induction of phenylalanine ammonia-lyase (PAL) activity because of cutting. As a result, the biosynthesis of polyphenols, which are substrates of polyphenol oxidase, was inhibited. This reduction of polyphenol synthesis caused the inhibition of the browning. Cinnamaldehyde treatment repressed the induction of PAL mRNA, while mild heat treatment did not repress its induction. The increase in microbes in cut lettuce treated with cinnamaldehyde was less than that treated with mild heat after 12 days.

KEYWORDS: Lettuce (*Lactuca sativa* L.), enzymatic browning, phenylalanine ammonia-lyase, cinnamaldehyde

■ INTRODUCTION

The shelf-time of cut vegetables and fruits, such as lettuce and apples, is often limited by enzymatic browning. During the storage of cut vegetables and fruits, their organoleptic characteristics are modified by the appearance of brown pigments. This browning is due to the oxidation of polyphenols by polyphenol oxidase (PPO; EC 1.14.18.1).[1,2] Formed quinones from polyphenols by the action of PPO are chemically or automatically polymerized to form brown pigments. After such fruits as apples and bananas are cut, the cut sections usually turns brown within 10 min to 1 h. On the other hand, it takes several days for the section of cut or shredded vegetables, such as lettuce[3,4] and cabbage,[5] to turn brown. This time lag is considered to be due to the *de novo* biosynthesis of polyphenols. Mature apples contain a sufficient amount of polyphenols for rapid enzymatic browning,[6] while lettuce leaves contain a much lower amount of polyphenols than apples.[7] The biosynthesis of polyphenols is hence considered to be a limiting factor in enzymatic browning for a cut vegetable, such as lettuce.

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), which converts L-phenylalanine to *trans*-cinnamic acid, is the rate-limiting enzyme of the phenylpropanoid pathway and is generally induced by wounding or cutting of plant tissue.[8] Such *o*-diphenols as dicaffeoyltartaric acid and chlorogenic acid (5-caffeoylquinic acid; CQA) formed by the phenylpropanoid pathway are oxidized by PPO to form brown pigments during the cold storage of cut lettuce. Several studies have described a relationship between PAL activity in lettuce leaves and browning.[3,9,10] Our group also showed that inhibitors of the shikimic acid and phenylpropanoid pathways repressed the browning of cut lettuce and that regulating polyphenol biosynthesis is essential to repress browning.[4] Heat shock (HS) or mild heat treatment at 50−60 °C repressed enzymatic browning of cut lettuce by suppressing the induction of PAL because of cutting.[11,12] The quality of cut lettuce treated by HS is better than that of nontreated cut lettuce,[13] but certain equipment and strict control of the temperature are essential for HS treatment. Another problem with mild heat treatment is an increase of microbes in treated vegetables during storage.[14,15]

Chemical methods to prevent enzymatic browning of cut lettuce have been also reported.[16,17] It is known that a PAL inhibitor, such as 2-aminoindan-2-phosphonic acid,[18] represses the browning of cut lettuce.[4,19] Although 2-aminoindan-2-phosphonic acid is a strong inhibitor, this compound is a reagent and cannot be used for food processing or storage. Recently, we found that *trans*-cinnamaldehyde, the major component of cinnamon, inhibited PAL activity and the browning of cut lettuce.[20] Cinnamaldehyde is generally recognized as safe or generally recognized as safe (GRAS).[21] We were not sure that the inhibitory activity of cinnamaldehyde against the browning of cut lettuce was attributed to the direct enzyme inhibition, although its enzyme inhibitory activity was definite. In this study, we compared the cinnamaldehyde and HS treatments on the enzymatic browning of cut lettuce during cold storage, because the mechanism of action of HS treatment against the browning has been extensively examined and well-established.[11,12,25] We also compared two treatments in terms of microbial growth during cold storage, because microbial growth in cut lettuce treated with mild heat tends to be promoted.[13−15] The purpose of this study is first to clarify the mechanism of action of cinnamaldehyde against the browning of cut lettuce during cold storage and second to show its microbiological merit compared to HS treatment.

■ MATERIALS AND METHODS

Lettuce and Storage. Commercially grown and harvested crisphead (iceberg) lettuce (*Lactuca sativa* L.) specimens were purchased from a retail shop in Tokyo in the 2003−2006 period and used for experiments without further storage. After 2−4 leaves were discarded, the next 10 uninjured leaves were removed and 2 × 4 cm mid-rib
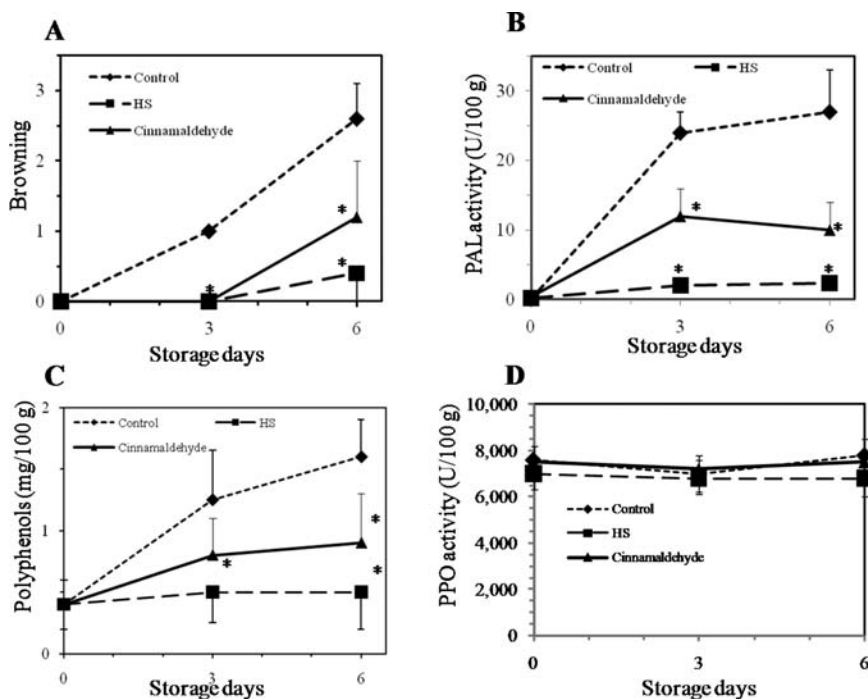
Figure 1. Effect of the cinnamaldehyde treatment to cut lettuce on (A) browning, (B) PAL activity, (C) polyphenol content, and (D) PPO activity during cold storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution for 30 min and washed with water twice before being wrapped with a plastic film and stored at 4 °C for 6 days. HS (50 °C for 90 s) treatment was performed as a positive control. (∗) Significant difference ($p < 0.05$) against the control ($n = 5$).

segments were excised, starting 1 cm from the base of the leaf. These segments were used as cut lettuce.

Treatment of Cut Lettuce. Cut lettuce (about 15 g) just after cutting was immersed in 150 mL of a 0.05% solution of *trans*-cinnamaldehyde (cinnamaldehyde treatment) or a related compound in a 1% ethanol solution for 30 min and washed with cold water (4 °C) twice. As a control, a 1% ethanol solution was used. For HS treatment, cut lettuce (about 15 g) was soaked in 150 mL of warm water at 50 °C for 90 s and then cooled by cold water at 4 °C. Each sample was wrapped in clear food-grade plastic film (Saran Wrap, Asahi Kasei, Tokyo, Japan) and stored at 4 °C for 9 or 12 days.

Reagents. *trans*-Cinnamaldehyde, *trans*-cinnamic acid, cinnamyl alcohol, α-hexylcinnamaldehyde, *o*-nitrocinnamaldehyde, and 3-phenyl-1-propanal were purchased from Wako Pure Chemical Industries (Osaka, Japan). α-Methyl-*trans*-cinnamaldehyde, α-amylcinnamaldehyde, and 2-methoxy-cinnamaldehyde were purchased from Sigma-Aldrich (St. Louis, MO). *trans*-Cinnamic acid was purchased from Nacalai Tesque (Kyoto, Japan).

Evaluation of Browning. The browning of the cut edges of the lettuce segments was evaluated visually. Scoring was in a range from 0 (no browning), 1 (slight browning), 2 (definite browning), to 3 (extreme browning).

Enzyme Extraction and Assay. The procedure by Siriphanich and Kader[23] was adapted to the extraction of PPO and PAL from lettuce, with some modifications. In a cold room (4 °C), the lettuce segments (about 15 g) were cut into small pieces with a knife and homogenized by a Polytron PT10/35 homogenizer (Kinematica, Inc., Lucerne, Switzerland) for 30 s with 3 times weights of a 0.2 M boric acid−NaOH buffer (pH 8.8) containing 1 mM dithiothreitol for PAL and of McIlvaine buffer (pH 6.5) for PPO. Each homogenate was filtered through four layers of cotton gauze, and the resulting filtrate was centrifuged at 12000g for 20 min. The supernatant obtained was used as a crude enzyme.

PAL activity was measured by a spectrometric method at 290 nm to detect the increase in cinnamic acid as a product.[23] The reaction solution consisted of 2.80 mL of a 0.5 M boric acid−NaOH buffer (pH 8.8), 0.40 mL of 10 mM L-phenylalanine, and 0.80 mL of the crude enzyme solution. A total of 1 unit of PAL activity was defined as the amount of enzyme that produced 1 μmol of cinnamic acid in 1 h at 40 °C.

PPO activity was measured by the spectrophotometric method at 325 nm to detect the decrease in CQA as the substrate.[24] The reaction solution consisted of 3.2 mL of McIlvaine buffer (pH 6.5), 0.4 mL of 50 μM CQA, and 0.4 mL of the crude enzyme solution. A decrease in the absorbance of 0.1/min at 30 °C was defined as 1 unit.

Polyphenol Determination. Polyphenols were extracted from lettuce samples and measured by the high-performance liquid chromatography (HPLC) methods as described before,[13] according to the method by Hisaminato et al.[4] Four polyphenols (CQA, 3,5-dicaffeolyquinic acid, dicaffeoyltartaric acid, and caffeoyltartaric acid) were measured from its peak area at 320 nm as CQA, and the sum of four polyphenols was regarded as the polyphenol amount.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Western Blotting. Crude extracts for PAL from cut lettuce stored for 4 days at 4 °C were separated by 10.0% SDS−PAGE, before proteins were transferred from the gel onto a nitrocellulose membrane by a semi-dry blotting apparatus (AE-6677P/S/N, ATTO Co., Tokyo, Japan). The membrane was subjected to immunodetection of PAL. Rabbit anti-PAL antiserum was prepared using PAL polypeptide (CDPLQKPKQDRYALRTSPQ)[25] conjugated with KLH protein by the S−S bond (Scrum Inc., Tokyo, Japan). After 0.2 mL of a keyhole limpet hemocyanin solution (about 10 mg/mL) was added to the rabbit antiserum, the formed precipitate was removed by centrifugation at 10000g for 10 min and the supernatant was used as an anti-PAL antibody (first antibody). After the blotted membrane was treated with the anti-PAL antibody and washed with phosphate-buffered saline containing Tween 20, the membrane was treated with a goat anti-rabbit antibody conjugated with peroxidase (second antibody; Takara Bio, Ohtsu, Shiga, Japan) and then incubated with ECL western blotting

detection reagents (GE Health Care Japan, Tokyo, Japan) according to the instructions of the manufacturer. Densities of bands were measured by ImageJ 1.41 [National Institutes of Health (NIH), Bethesda, MD].

Real-Time Polymerase Chain Reaction (PCR) of PAL mRNA. Frozen lettuce segments (about 0.3 g) were pulverized with pestle and mortar in liquid nitrogen. Total RNA was extracted and purified with RNeasy plant mini kit (Quiagen, Tokyo, Japan) according to the instructions of the manufacturer. Total RNA adjusted to about 300 ng in 10 μL was reverse-transcribed to cDNA using ExScript RT reagent kit (Takara Bio, Ohtsu, Shiga, Japan) according to the instructions of the manufacturer. Real-time PCR was performed with an ABI PRISM 7500 fast real-time system (Applied Biosystems, Carlsbad, CA) using SYBR Premix Ex Taq (Takara Bio, Ohtsu, Shiga, Japan). Primers were designed on the base of two PAL genes [PAL-1 (AF20030) and PAL-2 (AF411134)][25] of Romaine lettuce. 16S rRNA was used as an internal control. The forward and reverse primer sequences for PAL and 16S rRNA were as follows: PAL: forward primer (positions 1116−1135 in PAL-1 and positions 1089−1108 in PAL-2), 5′-ACGAAATGGACCGTTACAG-3′; reverse primer (positions 1215−1234 in PAL-1 and positions 1188−1207 in PAL-2), 5′-TTCCCT-CTCGATCATTTTGG-3′ and 16S rRNA: forward primer, 5′-TTGCCA-TCATTGAGTTTGGA-3′; reverse primer, 5′-GCATAAGGGGCAT-GATGA-

CT-3′. The sizes of the resulting amplicons of PAL and 16S rRNA were 113 and 91 bp, respectively. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 30 s.

Analysis of Microbes. Each lettuce sample (10 g) was homogenized with 90 mL of phosphate-buffered saline with 0.1% peptone by a homogenizer (Masticator PS, GSI Crenos, Tokyo, Japan). Colony forming units (CFUs) for aerobic microbes were measured using Nutrient agar (Kanto Chemical Co., Tokyo, Japan), which was incubated at 37 °C for 48 h.

Statistical Analysis. Data obtained in the experiment were analyzed by one-way analysis of variation (ANOVA) with Statcel software (OMS Publishing, Tokorozawa, Japan) on Excel 2003 (Microsoft, Redmond, WA).

■ RESULTS AND DISCUSSION

Inhibition of Enzymatic Browning, PAL Induction, and Polyphenol Synthesis of Cut Lettuce by Cinnamaldehyde. We first examined the inhibitory effect of *trans*-cinnamaldehyde treatment on the browning and PAL activity of cut lettuce during cold storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution, before being washed and stored at 4 °C. As shown in Figure 1A, the browning of cut lettuce was significantly inhibited during 6 days of storage, although the inhibitory effect was weaker than that of HS treatment, which was used as a positive control. The induction of PAL activity by cutting was repressed by cinnamaldehyde treatment (Figure 1B). The increase in polyphenols during cold storage after cutting was repressed by cinnamaldehyde treatment as well as HS treatment (Figure 1C). PPO activity was not inhibited by cinnamaldehyde (Figure 1D). These results show that cinnamaldehyde inhibited the induction of PAL activity because of cutting, which caused the inhibition of the biosynthesis of polyphenols, which are substrates of PPO, and that the browning was consequently inhibited.

Relationship between the Inhibition of PAL Activity and the Inhibition of Browning of Cut Lettuce by Cinnamaldehyde-Related Compounds. Because *trans*-cinnamaldehyde can be a PAL inhibitor,[20] we examined whether or not the inhibitory effect of cinnamaldehyde on the induction of PAL activity was due to the direct enzyme inhibition, although lettuce samples

Table 1. Inhibitory Effect of Cinnamaldehyde-Related Compounds against PAL Activity and Browning of Cut Lettuce[a]

compound	PAL inhibition (%)	browning
trans-cinnamaldehyde	100	−
α-methyl-*trans*-cinnamaldehyde	100	−
α-amylcinnamaldehyde	100	+
2-methoxy-cinnamaldehyde	82	−
α-hexyl-*trans*-cinnamaldehyde	15	+
o-nitrocinnamaldehyde	0	−
trans-cinnamic acid	0	−
cinnamyl alcohol	0	−
3-phenyl-1-propanal	0	−
acetaldehyde	0	+

[a] Each compound (10 μg/mL) was added to a PAL assay solution, whose PAL activity was measured by a spectrometric method ($n = 2$). Browning (0.05% solution) was visually estimated 3 days after cutting. +, similar to the control; −, similar to *trans*-cinnamaldehyde.
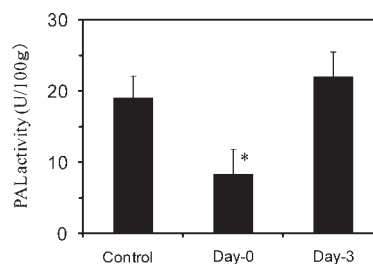

Figure 2. Effects of the cinnamaldehyde treatment on the PAL activity of cut lettuce after 3 days of storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution (1% ethanol) for 30 min (day 0) or 1% ethanol (control). After washing, it was stored at 4 °C for 3 days. After 3 days of storage, a part of control lettuce was immersed in 0.05% *trans*-cinnamaldehyde solution and washed (day 3). PAL activity of each sample was measured. (∗) Significant difference ($p < 0.05$) against the control ($n = 5$).

were washed with water after treatment. Table 1 shows the inhibitory effect of cinnamaldehyde-related compounds on PAL activity and their inhibitory activities against the browning of cut lettuce during storage. A part of cinnamaldehyde moiety seems to be essential to inhibit PAL activity because cinnamaldehyde, α-methyl-cinnamaldehyde, α-amylcinnamaldehyde, and 2-methoxy-cinnamaldehyde inhibited PAL activity, while cinnamic acid and cinnamyl alcohol did not inhibit PAL activity. However, the relationship between the inhibition against PAL activity and the inhibitory effect on the browning of cut lettuce was not apparent. Some compounds inhibited both PAL activity and the browning, while other compounds inhibited either PAL activity or browning. For example, 3-phenyl-1-propanal, cinnamyl alcohol, cinnamic acid, and α-nitrocinnamaldehyde inhibited the browning but not PAL activity. On the other hand, α-amylcinnamaldehyde inhibited PAL activity but not the browning.

This inconsistency suggests that the inhibitory effect of cinnamaldehyde on the browning was not directly due to the direct enzyme inhibition. To ascertain this, cut lettuce during storage was treated again with cinnamaldehyde, before the PAL activity was measured. If direct enzyme inhibition was the major mechanism for inhibition against the browning of cut lettuce, the
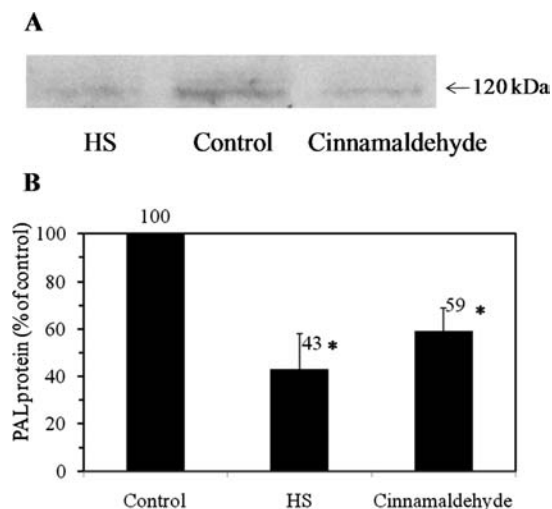
Figure 3. Effect of the cinnamaldehyde treatment on the amount of induced PAL protein in cut lettuce during cold storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution in 1% ethanol (cinnamaldehyde) or 1% ethanol (control) for 30 min. After washing, it was stored at 4 °C for 4 days. PAL proteins after 4 days of storage were (A) analyzed by western blotting—ECL analysis and (B) densitometerically determined. HS (50 °C for 90 s) treatment was performed as a positive control. (∗) Significant difference ($p < 0.05$) against the control ($n = 3$).

inhibitory effect of the second cinnamaldehyde treatment against the induced PAL activity was expected, because the induced enzyme should be similarly inhibited by residual cinnamaldehyde after washing. However, the PAL activity of cut lettuce treated with cinnamaldehyde at day 3 was not inhibited at all (Figure 2). Further, we could not detect cinnamaldehyde from a washed cut lettuce sample treated with cinnamaldehyde (data not shown). These data strongly suggest that the inhibitory effect of cinnamaldehyde on the browning was not due to the direct inhibition of PAL.

As latter descried, the inhibitory effect of cinnamaldehyde on the browning of cut lettuce during cold storage was due to the fact that cinnamaldehyde inhibited the induction of PAL protein by cutting at the level of mRNA. Therefore, it is considered that such compounds as α-amylcinnamaldehyde that inhibited PAL activity but not the browning might not inhibit the induction of PAL protein. Conversely, such compounds as 3-phenyl-1-propanal, cinnamyl alcohol, cinnamic acid, and α-nitrocinnamaldehyde that inhibited the browning but not PAL activity might inhibit the induction of PAL protein. Because 3-pheyl-1-propanal, cinnamic acid, and cinnamyl alcohol inhibited the browning but did not inhibit the enzyme activity, a phenyl group connected with a three carbon chain might be essential to inhibit the browning. However, we do not know why α-amylcinnamaldehyde and α-hexyl-cinnamaldehyde did not inhibit the browning, although cinnamaldehyde, α-methyl-cinnamaldehyde, and α-methoxy-cinnamaldehyde inhibited the browning. Further investigations will be needed to understand the structure—activity relationship of cinnamaldehyde-related compounds in terms of the inhibitory effect on the browning of cut lettuce.

Effect of Cinnamaldehyde and HS Treatments on the PAL Protein Level. Because the induction of PAL activity during cold storage was repressed by cinnamaldehyde treatment, the PAL protein level was examined. HS treatment was used as a positive
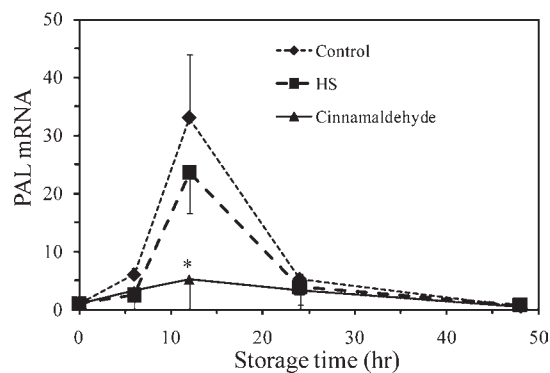

Figure 4. Effect of the cinnamaldehyde treatment on PAL mRNA level in cut lettuce during cold storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution in 1% ethanol (cinnamaldehyde) or 1% ethanol (control) for 30 min. After washing, it was stored at 4 °C. The PAL mRNA level was measured for 48 h by the real-time PCR method. The amount of PAL mRNA of a control cut lettuce at 0 h was defined as 1.0. HS (50 °C for 90 s) treatment was performed as a negative control. (∗) Significant difference ($p < 0.05$) against the control ($n = 2$).
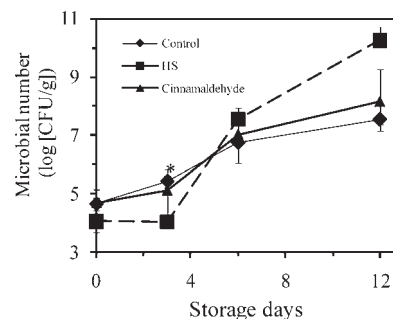

Figure 5. Comparison of cinnamaldehyde and mild HS treatments on bacterial growth in cut lettuce during cold storage. Cut lettuce was immersed in a 0.05% *trans*-cinnamaldehyde solution in 1% ethanol (cinnamaldehyde) or 1% ethanol (control) for 30 min. After washing, it was stored at 4 °C. Mild HS treatment was performed at 50 °C for 90 s. CFUs for aerobic microbes were measured by the agar plate method.

control, because it was known to repress the induction of PAL protein.[22] As shown in Figure 3, the PAL protein level of cut lettuce treated with cinnamaldehyde was decreased to about 60% of the control. This repression seemed to be weaker than that of HS treatment.

Effect of Cinnamaldehyde and Mild HS Treatments on the PAL mRNA Level. Because cinnamaldehyde treatment repressed the induction of PAL activity and protein level, its effect on the PAL mRNA level was examined. While the PAL mRNA level was definitely raised at 12 h after cutting in the control lettuce, this induction was significantly repressed by the cinnamaldehyde treatment (Figure 4). HS treatment did not repress this induction. Campos-Vargas et al.[22] reported that HS treatment did not repress the induction of PAL mRNA but inhibited the translation of mRNA into PAL protein. Because the inhibitory effect of cinnamaldehyde on the browning of cut lettuce was weaker than HS treatment, the inhibitory effect of HS treatment on PAL protein synthesis from PLA mRNA is considered to be definite and strong.

Effect of the Cinnamaldehyde Treatment on Bacterial Growth during Cold Storage. Although HS treatment is a very useful method to regulate the browning of cut lettuce during cold

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storage, it is reported that HS treatment increases microbes in treated vegetables during storage.[13−15] We then compared the growth of microbes in the cut lettuce treated with cinnamaldehyde and mild heat. Although the number of microbes of cut lettuce was decreased by HS treatment at day 3, the number significantly increased at day 12 (Figure 5). On the other hand, the number of microbes of the cut lettuce treated with cinnamaldehyde was almost similar to that of a control lettuce. We further inoculated *Staphylococcus aureus*, *Salmonella* spp., and *Escherichia coli* on cut lettuce and examined the change of the number of these bacteria. These foodborne disease bacteria did not grow at 4 °C, and the bacterial number in cut lettuce treated with cinnamaldehyde during 12 days of cold storage was almost similar to those of the control cut lettuce (data not shown).

In conclusion, cinnamaldehyde repressed the induction of PAL mRNA because of cutting and inhibited the polyphenol biosynthesis and browning during cold storage. Although the inhibitory effect of cinnamaldehyde was weaker than that of HS treatment, the increase of microbes in cut lettuce treated with cinnamaldehyde during cold storage was less than that treated with mild heat.

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■ ACKNOWLEDGMENT

■ ABBREVIATIONS USED

PPO, polyphenol oxidase; PAL, phenylalanine ammonia-lyase; CQA, chlorogenic acid; HS, heat shock

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