Effect of Kiwifruit Juice on Beef Collagen

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Summary The objective of this study was to examine the effect of kiwifruit juice on collagen in meat during cooking processes and to clarify the difference in susceptibility to protease digestion by kiwifruit juice between collagen domains under different conditions. In addition, the effect of pre-treatment with kiwifruit juice on collagen in meat was also examined. Kiwifruit juice can degrade denatured collagen, but it can not cleave the triple helical domain of collagen. Thus, kiwifruit juice does not have collagenase activity. On the other hand, the cross-linked subunits of acid-soluble collagen were converted to monomeric subunits by kiwifruit juice treatment at acidic pH, suggesting that the globular domains, which cross-links preferentially occur, can be degraded by kiwifruit juice. The pre-treatment with kiwifruit juice significantly decreased the shear force of connective tissue in comparison with other pre-treatments without protease activity, but inversely increased the liberation of collagen-related peptides in the outer solution by heating processes at 50 and 70°C or by a shorter heating time at 100°C. This can be explained by the protease-mediated degradation of globular domains. However, this effect was not observed with a prolonged heating period at 100°C, and the liberation of collagen-related peptides by pre-treatment with kiwifruit juice at 100°C was less than that at 70°C for all heating periods. Thus, it can be suggested that the pre-treatment with kiwifruit juice might be useful in meat softening under vacuum-cooking and grilling, but not under stewing.

Key Words collagen, gelatin, meat, kiwifruit juice, tenderization

Texture is one of several important factors in determining the quality of meat dishes. Meat consists of muscular, connective, and adipose tissues. Collagen (type I), a major constituent of intramuscular connective tissue, has a triple helical domain and globular domains which are referred to as telopeptides (1). The collagen molecules assemble in fibrils and are immobilized by cross-links which are preferentially formed between the triple helix and globular domains. Some cross-links are labile to heat and acid treatments, thus some collagen can be solubilized by heat and acid treatments. Heating treatment also converts the triple helix structure to the globular structure. The heat-induced globular form is referred to as gelatin. The content and nature of collagen and its cross-links markedly affect the toughness of meat (2). Thus, it can be considered that the degradation and structural change of collagen in the cooking process affect the meat texture.

Collagenase has been defined as an endoprotease which can cleave the triple helix structure of collagen (3, 4). On the other hand, the globular domains and heat-denatured collagen, i.e. gelatin, can be degraded with a variety of animal, plant or microbial proteases (1). Fruits and rhizomes of plants frequently have proteases of high activity, which are mostly classified as a cysteinyl type. There have been some reports about the collagenase activity in plants such as kiwifruit and ginger (5–7). In these studies, however, little attention was paid to the difference in susceptibility to proteolytic digestion between the domains of collagen as well as between collagen and gelatin. Thus, it remains unclear whether such plants have collagenase activity.

Crude extracts from fruits and rhizomes or their slices have been practically used as meat tenderizers. It has been demonstrated that proteases in such preparations play a significant role in the tenderization of meat, particularly in the degradation of myofibrillar protein (8–15). There have been some studies suggesting the degradation of collagen in meat (12–14). On the other hand, it has also been reported that meat was tenderized with pre-heated kiwifruit juice whose protease activity was inactivated (15). This suggests that some factor besides protease in kiwifruit juice can also tenderize meat. Thus, the contribution of collagen degradation by plant extract to meat tenderization in the cooking process remains to be explicated.

In previous studies, we reported that neither six cysteinyl proteases in kiwifruit nor two major proteases corresponding to actinidin [EC 3.4.22.14], had collage-

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Abbreviations: ASC, acid-soluble collagen; PSC, pepsin-solubilized collagen; ISC, insoluble collagen; PAGE, polyacrylamide gel electrophoresis; E-64, l-3-carboxy-2,3-trans-epoxypropionyl-leucylamide(4-guanidino)butane; HYP, hydroxyprolin.
nase activity (16, 17). However, it is possible that other proteases in kiwifruit may exert different substrate specificity. The objective of this study is to clarify the action of kiwifruit juice on native collagen with or without globular domains and on gelatin extracted from collagen with boiling water. In addition, the effect of pretreatment with kiwifruit juice on collagen in meat during the cooking process was also examined.

**MATERIALS AND METHODS**

1. **Preparation of kiwifruit juice.** Kiwifruit (*Actinidia chinensis*) was obtained from a local fruit market. The kiwifruit was peeled, mashed, and squeezed through a cotton cloth to obtain juice. The juice was immediately used to minimize the autoysis of kiwifruit proteases (16). To inactivate the proteases, part of the kiwifruit juice was either mixed with a final concentration of 0.1 mM E-64 (Peptide Institute, Osaka, Japan) or heated in a microwave for 2 min at 500 W.

2. **Reaction of kiwifruit juice with collagen.** Acid-soluble, pepsin-solubilized and insoluble collagens prepared from beef shank by the method of Sato et al. (18) were designated as ASC, PSC, and ISC, respectively, and were used as substrates hereinafter. In order to avoid the heat-denaturation of collagen, their preparation was always done below 10°C. ASC consists of globular and triple-helix domains. Since the globular domains are degraded by pepsin treatment, PSC consists exclusively of triple helix domain. ISC, which remained in an insoluble form after pepsin digestion, is considered collagen cross-linked between helical domains. Hence ISC was washed with cold distilled water and then homogenized in water to yield a suspension. These PSC, ASC and ISC preparations were dialyzed against 50 mM citrate-HCl buffer (pH 3.0), 50 mM citrate buffer (pH 5.0), or 50 mM phosphate buffer (pH 7.0), and subjected to the reaction with kiwifruit juice. Alternatively, aliquots of the preparations were pre-heated at 30, 50 or 80°C for 10 min, in which the collagen concentration ranged from 1.7 to 5.0 mg/mL.

To 1 mL of such a collagen solution were added 10 μL of kiwifruit juice, 10 μL of 1 mM dithiothreitol (DTT) as a cysteinyl protease activator, and 100 μL of 100 mM calcium chloride as a metallo-type collagenase activator, followed by incubation at 10°C to avoid the heat-denaturation of collagen. The reaction was terminated by adding 20 μL of 1 mM E-64 to 20 μL of the above mixture.

3. **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)** SDS-PAGE was performed by using 10.0 or 12.5% polyacrylamide gel by the method of Laemmlli (19). The molecular mass markers were obtained from TEFICO (Nagano, Japan). The sample solution was heated in a boiling water bath for 2 min to dissociate the collagen molecules into subunits. After electrophoresis, the gel was stained with Quick CBB (Wako, Osaka, Japan) or Sil-Best Stain (Nacalai Tesque, Kyoto, Japan). Since there was a linear relationship between the actual collagen content and the densitometric area of the collagen band on SDS-PAGE (20), the intensity of each stained band was measured using a Densitograph lane analyzer ver. 3.0 equipped with a Printgraph AE6915 (ATTO, Osaka, Japan).

4. **Measurement of shear force for the connective tissue.** Connective tissues were prepared from beef shank blocks (15×15×15 mm) by the method of Sato et al., and washed with cold distilled water (18). The connective tissue (2 g) was cut into 5×5×10 mm pieces and immersed in 1 mL of kiwifruit juice, pre-heated kiwifruit juice, 50 mM citrate buffer (pH 3.2): the pH of which corresponds to that of kiwifruit juice) or distilled water at 10°C for 30 min.

The shear force of each sample was measured by the method of Ando using a rheometer NRM-2010F-CW (Fudo, Tokyo, Japan) equipped with a razor-edge (21). The connective tissue was cut in a direction orthogonal to the collagen fibers.

5. **Liberation of collagen-related peptide from beef shank by treatment with kiwifruit juice.** Beef shank (fibrous part. 10 g: 2 g×5) was immersed in 5 mL of kiwifruit juice, pre-heated kiwifruit juice, 50 mM citrate buffer (pH 3.2) or distilled water at 10°C for 30 min. The beef shank pre-treated with each solution was mixed with 50 mL of distilled water pre-heated at 50, 70 or 100°C, and incubated at 50, 70 or 100°C for 10, 30 or 60 min. After incubation, the outer solution was collected. The amount of collagen-related peptides liberated from the beef shank was assessed by measurement of the hydroxyprolin (HYP) content after acid-hydrolysis with 6 N HCl at 130°C for 3.5 h. HYP was determined by the method of Woesnner (22). In some cases, the cooked meat was homogenized with the outer solution and centrifuged to obtain the supernatant, which was analyzed for HYP as described above. The supernatant contained essentially the same content of collagen-related peptides as did the outer solution.

6. **Statistical analysis.** The difference among four immersed samples in the shear force or in the liberation of collagen-related peptides was evaluated by the Tukey-Kramer test using StatView™ 5.0 (SAS Institute Inc.)

**RESULTS AND DISCUSSION**

1. **Effect of kiwifruit juice on triple helical domain of collagen**

Figure 1 shows the SDS-PAGE patterns of PSC treated with kiwifruit juice at pH 3.0. At reaction temperatures of 50 and 80°C, the collagenous bands disappeared extensively, but no significant change was observed at 10 or 30°C. Furthermore, PSC preparations pre-heated at 50 and 80°C were also extensively degraded by treatment with kiwifruit juice, even at a reaction temperature of 10°C (Fig. 2). Similar results were obtained when heat-denatured ISC and ISC at 50 or 80°C were used as substrates (not shown). This degradation of collagen did not occur when E-64 had been added to the reaction mixture (Fig. 2). These results indicate that the structural change in collagen by heating at above 50°C explains the reason for the extensive disappearance of collagenous bands caused by cysteinyl protease in kiwifruit juice, because the gelatinization temperature of...
dissolved beef collagen is approximately 40 °C (23). Therefore, the degradation of collagen observed at 50 and 80 °C was not attributed to cleavage of intact triple helix domains but to the degradation of heat-induced gelatin (Figs. 1 and 2). Similar results were obtained at pH 5.0 or 7.0 (not shown). These results indicate that kiwifruit juice exhibits no collagenase activity in the pH range of 3 to 7.

Several authors have reported that plant extracts including kiwifruit have collagenase activity (5–7). In these reports, apparent collagenase activity is measured above 50 °C, at which point collagen has been converted to gelatin (5, 6). It has also been reported that the optimum temperature for the relevant collagenase is 60 °C and the activity is maintained at 80 °C (5). In another study, the enzymatic reaction was terminated by heat treatment in SDS solution without any protease inhibitor (7). We found that the substrate can be rapidly degraded by purified kiwifruit protease even during heat treatment with SDS (16). These findings imply that all experiments should be performed below 37 °C, at which collagen maintains its triple helical structure, to assess collagenase activity. Thus, the activity detected in the above studies should be referred to as gelatinase activity.

We previously reported that the two major proteases of kiwifruit sharing 30.8 and 46.7% in activity had no collagenase activity (16, 17). The present results also reveal that whole kiwifruit extract can not cleave the triple helix domain of collagen in the presence of calcium chloride or DTT.

2. Effect of kiwifruit juice on globular domains of intact acid-soluble collagen

To examine the effect of kiwifruit juice on the globular domains of collagen, intact ASC was used as a substrate. At both pH 3.0 and 5.0, no significant amounts of degradation products lower in molecular weight than α-chain of collagen were observed, implying that no collagenase activity exists in kiwifruit juice (Fig. 3). On the other hand, cross-linked subunit chains (β- and γ-chains) were converted to α-chain at pH 3.0. The staining intensity of β- and α-chains after reaction for 2 h decreased and increased to 51 and 199% of the initial values, respectively (Fig. 3A). This result suggests that kiwifruit juice containing some proteases might degrade the globular domains which are involved in

![Fig. 1 SDS-PAGE patterns of PSC reacted with kiwifruit juice at varying reaction temperatures (10–80 °C). The reaction was carried out with 1 mL of collagen in 50 mM citrate buffer (pH 3.0) for 2 h. The reaction was terminated by treatment with E-64. After electrophoresis, the gel was stained with CBB except in the case of using kiwifruit juice. The gel-loaded kiwifruit juice was stained with Sil-Best stain. KJ: kiwifruit juice. Mr: molecular mass markers.](image1)

![Fig. 2 SDS-PAGE patterns of pre-heated PSC reacted with kiwifruit juice at 10 °C. (A) The non-heated PSC and PSC pre-heated at 30, 50, or 80 °C for 10 min were used as substrates. The reaction was performed at 10 °C with 1 mL of collagen in 50 mM citrate buffer (pH 3.0) for 0, 1 and 2 h. The reaction was terminated by treatment with E-64. After electrophoresis, the gel was stained with CBB. (B) Kiwifruit juice was mixed with E-64 before the reaction to inactivate the protease. The reaction was performed for 2 h. The other conditions were the same as in (A).](image2)
intra- and intermolecular cross-linking. At pH 5.0, a significant decrease was observed for the highly cross-linked chains relative to γ-chains, but not for β-chains. The staining intensities of β- and α-chains after 2 h were 12.4% and 98% of the initial values, respectively (Fig. 3B). Similar results were also obtained at pH 7.0 (not shown). Such changes in digestion did not occur in the presence of E-64.

Ohyama et al. reported that the globular domains of collagen are degraded by purified kiwifruit protease (pH 3.0), but not by purified ginger protease (pH 6.0) (24). However, they did not describe whether this difference is due to the pH-activity profiles of the proteases or to structural changes in collagen molecules. We confirmed that squeezed ginger juice as well as kiwifruit juice degraded globular domains at pH 3.0 but not at pH 5.0 or 7.0 (not shown). Our previous study has shown that the two major kiwifruit proteases have high activities for S-3-trimethylaminopropyl-lysozyme and N-α-carbobenzyoxyl-lysine p-nitrophenyl esters at neutral pH, and that they can degrade neither ovalbumin nor bovine serum albumin with a disulfide bond-reinforced tight tertiary structure but can readily degrade casein free of such disulfide bonds (17). Collagen molecules associate into a supermolecular assembly and form fibrils at neutral pH. Taken altogether, it is reasonable to consider that the degradation of globular domains is triggered by pH-dependent structural changes in collagen molecules but that the globular domains are usually buried in the fibrils and protected from kiwifruit proteases at neutral pH.

3. Effect of kiwifruit juice on intact insoluble-collagen

As shown in Fig. 4, the reaction of kiwifruit juice with intact ISC even at pH 3.0 yielded no significant change in SDS-PAGE patterns. Similar results were obtained at pH 5.0 and 7.0 (not shown). ISC is cross-
linked between helical domains so as to maintain a fibrillar structure even at acidic pH. Thus, ISC is resistant to kiwifruit proteases because of its rigid supramolecular structure.

4. Effect of kiwifruit juice on shear force in connective tissue

As shown in Figs. 1–4, protease activity in kiwifruit juice can be suppressed when such a cysteinyl protease inhibitor as E-64 is added in excess to the reaction mixture. However, in this experiment, a larger amount of kiwifruit juice was used than that in the in vitro digestion of collagen (Figs. 1–4). As kiwifruit juice contains significant amounts of thiol compounds which potentially bind to E-64, it was difficult to completely suppress the activity of kiwifruit proteases by addition of E-64. Thus, the protease activity of kiwifruit juice could be corrected by the use of pre-heated kiwifruit juice without any protease activity.

The pre-treatment with acidic solutions devoid of protease activity, i.e., citrate buffer or pre-heated kiwifruit juice, decreased the shear force of connective tissue (Fig. 5). As some intermolecular cross-links between collagen subunits are susceptible to acid treatment (1), it can be assumed that the shear force of the connective tissue was weakened by the cleavage of such cross-links.

In practice, the pre-treatment with kiwifruit proteases significantly decreased the shear force of the connective tissue in comparison with the case of the acid-treatment. As demonstrated above, the kiwifruit proteases could cleave the collagen globular domains in which cross-links were predominant. Thus, it can be concluded that the protease action on the globular domains brings about a decrease in the shear force as well.

There have been many reports indicating that the treatment with various plant proteases affects the texture of cooked meat (8–15). The present study suggests that the degradation of collagen globular domains by kiwifruit proteases might improve the mechanical strength of raw meat.

5. Effect of pre-treatment with kiwifruit juice on liberation of collagen-related peptides from beef in following heating process

As shown in Fig. 6, the pre-treatment with kiwifruit juice alone could liberate only a negligible amount of collagen-related peptides from the beef shank at 10°C, despite the decreased strength of the connective tissue. This can be feasibly explained by the degradation of collagen globular domains poor in HYP that actually occurs by pre-treatment at 10°C.

When the beef shank was pre-treated with distilled water, citrate buffer (pH 3.2) or pre-heated kiwifruit juice, the liberation of collagen-related peptides increased in quantity with heating time and temperature. The heating in acidic solutions yielded a higher liberation of collagen-related peptides than that in distilled water. Since collagen molecules were more readily gelatinized by heating in acidic conditions than in neutral conditions (25), it can safely be said that collagen has emerged from meat connective tissue by cleavage of the cross-links during heat and acid treatment.

By heating at 50, 70 and 100°C for a short time, the pre-treatment with kiwifruit juice bearing active proteases led to a considerable liberation of collagen-related peptides in the outer solution. The protease activity of kiwifruit juice was observed at 80°C with denatured collagen as substrate, as described above, while that with casein as substrate was maintained at a level of 80% even after heating at 55°C for 3 h (24).
Thus, this enhanced liberation of collagen-related peptides could be interpreted in the following two ways: first, gelatin induced by the heat treatment might be degraded into small peptides by kiwifruit proteases, resulting in the enhanced liberation of collagen-related peptides; secondly, the degradation of globular domains by kiwifruit proteases proceeded during the pre-treatment and heating process. The resultant collagen without globular domains during the pre-treatment might be more easily liberated from the connective tissue by the heating process. The liberation of related peptide by a short-term heating at 100°C might be attributed to the degradation of collagen in the pre-treatment rather than in the heating process, because kiwifruit proteases are inactivated at 100°C. Characterization of collagen-related peptides liberated by kiwifruit juice and following heat treatments is required for elucidation of these hypotheses.

On the other hand, the further enhancement of collagen-related peptide liberation by kiwifruit proteases was no longer observed in a prolonged heating period at 100°C (60 min). In addition, the liberation of collagen-related peptides by the pre-treatment with kiwifruit juice bearing active proteases at 100°C was less than the cases of all heating periods at 70°C (p<0.01), which might be due to the inactivation of kiwifruit proteases. Therefore, the liberation of collagen-related peptides during the heating process with kiwifruit juice depends on physicochemical structural changes in collagen molecules and inactivation/activation of proteases by heating.

We conclude on the basis of these results that proteases in kiwifruit juice are fairly effective for the tenderization of meat by degrading collagen non-helical domains and/or inducing gelatin under cooking conditions at relatively low temperatures (e.g., vacuum cooking) or a short-term cooking process (e.g., grilling), but not so effective under a long-term cooking process at high temperatures (e.g., stewing). At present, further studies on the optimum tenderization of meat by kiwifruit juice are in progress.

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