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# Inhibition of Transglutaminase and Microbial Transglutaminase Activity by Garlic

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**Abstract** Ground garlic inhibited the cross-linking reaction of myosin and incorporation of monodansylcadaverine (MDC) in salted Alaska pollack surimi catalyzed by transglutaminase (TGase). The component responsible for the inhibition was a thermostable, low molecular weight compound. The component also inhibited microbial transglutaminase (MTGase). The inhibition by garlic was reversibly recovered upon addition of 2-mercaptoethanol. The inhibitory component was therefore hypothesized to contain sulfhydryl groups within its structure. Alliin itself did not inhibit the cross-linking reaction. However, the addition of alliin together with garlic increased the inhibition. This result suggested that compounds derived from alliin was responsible for the inhibition of TGase activity.

**Keywords:** garlic, myosin, surimi, cross-linking, transglutaminase, alliin

## Introduction

It is well known that certain Korean spices such as garlic possess health-promoting attributes. In particular, the antithrombotic (1), antimicrobial (2, 3), antioxidant (4, 5), and antihypertensive (6) action of garlic has been well demonstrated. In a previous paper, we reported that the addition of ground garlic to surimi significantly suppressed the setting effect, as the breaking strength of the thermal gel was increased by preheating the salted surimi at 25°C prior to heating at high temperature (7). The reaction was accompanied by the inhibition of the cross-linking reaction of myosin during the pre-heating process. The involvement of transglutaminase (TGase, E.C.2.3.2.13) in the cross-linking reaction of myosin heavy chain (HC) molecules in the setting process has been proposed (8, 9). This result suggested that ground garlic may contain bioactive compounds that inhibit this cross-linking reaction. One of the best known of these bioactive components is allicine which is produced by converting alliin, a sulfhydryl compound by an enzymatic reaction (10, 11). In this study, we investigated the mechanism of the inhibition of myosin cross-linking by ground garlic occurring during the incubation of salted surimi.

## Materials and Methods

**Materials** Fresh garlic was purchased from a local market in Hakodate, Japan. Frozen surimi (FA grade) of Alaska pollock (*Theragra chalcogramma*) was obtained from Nissui Co., Ltd. (Tokyo, Japan). The surimi contained 4% sucrose, 4% sorbitol, and 0.25% sodium polyphosphate. The water and protein content were 75.3 and 17%, respectively. Microbial transglutaminase (MTGase) was a kind donation by Ajinomoto Co., Ltd. (Tokyo, Japan). Monodansylcadaverine (MDC) and alliin were purchased from Sigma (St. Louis, MO, USA).

**Preparation and incubation of salted surimi** Frozen surimi stored at -40°C was transferred to a cold room (4°C) prior to use. Diced surimi was chopped on a food processor (Speed Cutter MK-K7; Matsushita Electric Industrial Co., Ltd., Osaka, Japan). 25% cold water was added together with 2.5%(w/w) NaCl to the processed surimi. The mixture was further chopped 8 times for 30 sec with 30 sec intervals to make a homogeneous salted surimi paste. At this stage, ground garlic and other compounds were added. To study the incorporation of MDC catalyzed by TGase, 2 mM MDC was added to the ground surimi.

The samples were then wrapped with polyvinyledene film were transferred to a temperature-controlled water-bath (NTT-2400; Eylea, Tokyo, Japan) held at 25°C.

**Analysis of cross-linking and MDC incorporation reactions** Samples (ca. 0.1 g) were taken from the pre-heated salted surimi prepared as above at certain period of incubation. These samples were then to 20 volumes of a solution containing 8 M urea, 2% SDS, 2% 2-mercapto-ethanol, and 20 mM Tris-HCl (pH 8.0) (SDSUM-solution) to stop the reaction, and to dissolve the surimi. The mixture was stirred overnight at room temperature to dissolve the surimi completely (12). Myosin cross-linking or MDC incorporation was studied by using sodium dodecysulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was carried out on 3% polyacrylamide - 0.5% agarose gel containing 0.1% SDS (13). To detect the cross-linked products, protein bands separated on the electrophoresis gel were stained with Coomassie Brilliant Blue R-250. To study the incorporation of MDC by the proteins in surimi, the fluorescent bands in the gel pattern were visualized under ultraviolet light illumination. Digital images of the fluorescent band patterns were photographed

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using a digital camera (C-4040 Zoom; Olympus, Tokyo, Japan).

**Fractionation of inhibitory compounds by dialysis** Ground garlic was dialyzed against $H_2O$ overnight using dialysis tubing (pore size 12,000; Spectrum Laboratory Inc., Gardena, CA, USA) to separate low molecular weight components from the ground garlic. Inner and outer solutions were separately collected, and were lyophilized. The lyophilized powder was dispersed into the original volumes of $H_2O$ used for dialysis to keep the concentration constant as those for ground garlic preparations.

## Results and Discussion

**Inhibition of MDC incorporation to myosin by garlic** Previous work showed that the myosin cross-linking reaction in surimi preparations was inhibited upon addition of ground garlic and it was then hypothesized that garlic inhibits TGase activity (7). Inhibition of the cross-linking reaction by garlic was confirmed by using a TGase assay system of MDC incorporation. The appearance of fluorescent bands after SDS-PAGE of surimi-garlic extracts incubated with 2 mM MDC is direct evidence of MDC incorporation by HC (Fig. 1A and B). In the absence of garlic, fluorescence intensity due to myosin heavy chain (HC) and myosin HC dimers increased with duration at 25°C. Figure 1A clearly shows that MDC addition inhibited the cross-linking reaction and a small amount of myosin HC polymers were produced. As MDC (amine) is a substrate for the TGase, high concentrations of the compound preferentially bind to the enzyme leading a low probability of the enzyme to bind myosin. Conversely, low concentrations of MDC facilitate increased myosin cross-linking. Addition of garlic almost completely suppressed the cross-linking reaction (Fig. 1A). Garlic also completely inhibited the incorporation of MDC to myosin HC catalyzed by TGase. As ground garlic inhibited not only myosin HC cross-linking but also MDC incorporation, we concluded that TGase activity in surimi was inhibited by compounds in garlic.

**Thermal stability of inhibitory compounds in garlic** To study the properties of the components that inhibited TGase activity, we first investigated the stability of the compounds. Ground garlic was heated for 30 min at various temperatures up to 80°C, and their inhibitory effect was measured (Fig. 2). Unheated garlic almost completely inhibited the cross-linking reaction and myosin HC content remained unchanged during the incubation. Addition of garlic heated for 80°C also inhibited the cross-linking reaction. The same inhibitory activity was detected when the experimental sample heated in boiling water for 10 min. These results clearly demonstrate that the inhibitory component was thermostable, and its effect was not diminished by heating. The inhibitory effect of ground garlic was also observed even after storage for one month in refrigerator (data not shown). In the previous paper, we described that dried commercial garlic powder showed the ability to inhibit this cross-linking reaction. We concluded the putative inhibitory component is very stable.

**Fractionation of the inhibitory compounds in ground garlic** The very high thermostability of the inhibitory compound suggested that the compound was unlikely to be a high molecular weight protein. We wondered therefore if the component is a low molecular weight compounds. To investigate this possibility, ground garlic was dialyzed against water where low molecular weight components moved into the outer solution and high molecular weight compounds were retained inside the dialysis tubing. The inner and outer fractions and ground garlic were mixed with surimi, and their effects on the cross-linking reaction were studied (Fig. 3). In surimi preparations containing no garlic, myosin HC disappeared from the protein band patterns on gels after an incubation period of 4 hr indicating a formation of highly cross-linked products. The incubation also produced an apparent

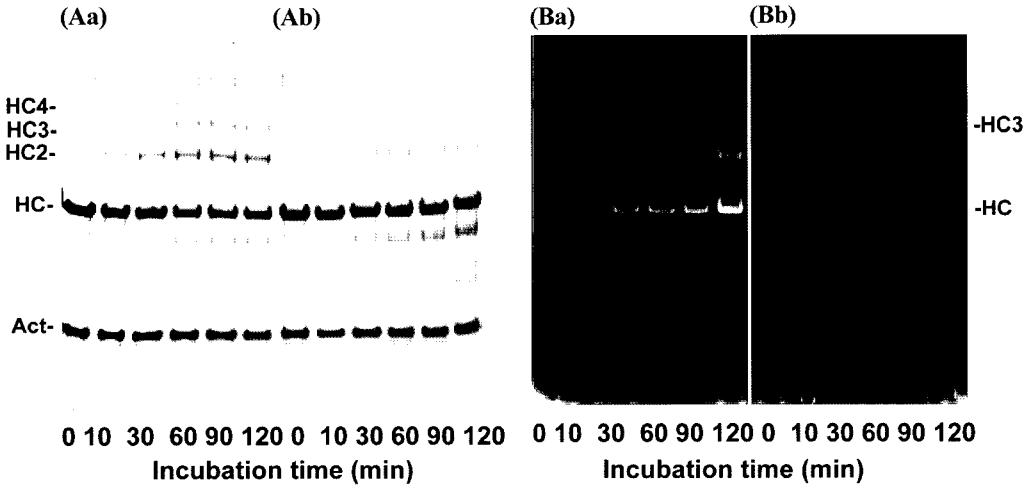


**Fig. 1. Inhibition of MDC incorporation into myosin by garlic.** Surimi was ground with 0.5 M NaCl and incubated at 25°C with 2 mM MDC in the absence (a) or presence (b) of 5% ground garlic. Protein bands stained with Coomassie Blue (A) and protein band fluorescence derived from incorporated MDC under UV lamp (B) were shown. HC, HC2, HC3, and HC4 are myosin heavy chain monomer, dimer, trimer, and tetramer, respectively.

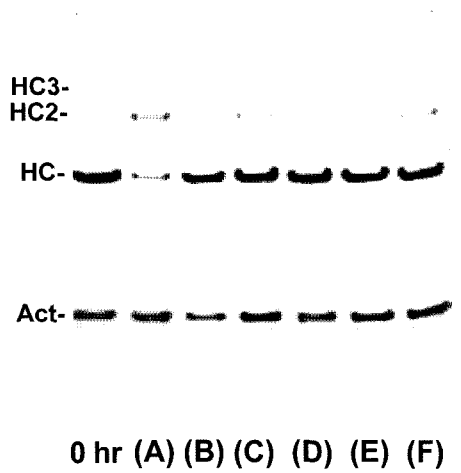


**Fig. 2. Thermal stability of the putative inhibitory component of garlic.** Ground garlic was heated for 30 min at 50 (C), 60 (D), 70 (E), or 80°C (F). Surimi paste was mixed with the heated garlic (5%) and incubated at 25°C for 1 hr. Sample incubated without garlic (A), and with unheated garlic (B) were also analyzed. 0 Hr denotes the pattern without incubation.

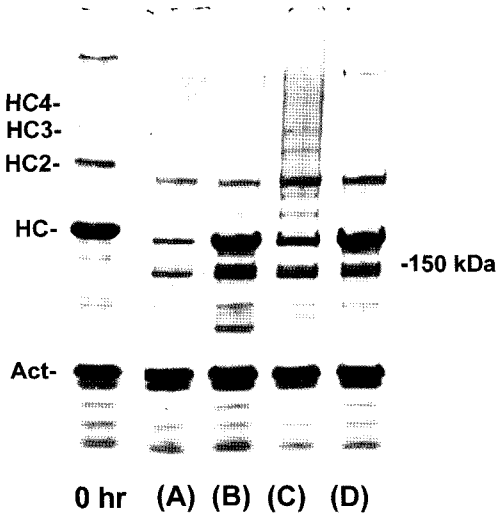


**Fig. 3. Fractionation of the putative inhibitory component of garlic.** Ground garlic was dialyzed against water overnight. Inner and outer solutions were lyophilized separately. They were dissolved in the original volumes of water for the ground garlic used. Surimi paste was mixed with ground garlic before dialysis (B), outer fraction of the dialysis (C), and inner fraction of the dialysis (D). The mixtures were incubated at 25°C for 4 hr. (A) shows the protein band pattern of surimi incubated without garlic. 0 Hr denotes the pattern without incubation.

degradation compound with a size of 150 kDa. The fragment was most likely produced by proteases in surimi (14). Larger amounts of this fragment appeared to be produced during prolonged incubations at 25°C. Surimi preparations that contained 5% ground garlic retained a high HC content even after 4 hr incubation along with the 150 kDa fragment. These results indicate that the compounds in garlic did not inhibit observed enzymatic degradation of HC. When the outer fraction was added, the protein band pattern was very similar to those produced by surimi preparations containing ground garlic indicating that this outer fraction contained compounds that inhibit the cross-linking. Again, the putative 150 kDa degradation fragment was observed in these samples. As previously reported, cross-linked products between myosin HC and the 150 kDa fragment, as well as between two 150 kDa fragments in the pattern migrating between myosin HC and HC dimers were observed (14). The addition of the concentrated inner dialysis fraction slightly increased the remaining HC content. This increase might be due to the contamination compounds originating from the outer solution because the inner fraction always contains some outer solution after equilibrium in the dialysis procedure. Based on these results, we concluded that the putative inhibitor for TGase present in garlic is a low molecular weight compound.

**Reversibility of the inhibition** Garlic contains many low molecular weights compound. It is well known that some of functional compounds in garlic are compounds that possess sulfhydryl (SH) groups. As TGase is SH-containing enzyme, we tested the possibility that the sulfhydryl group is involved in the inhibition of HC cross-linking by garlic. TGase activity should be inhibited if SH groups are essential for the normal catalytic activity in TGase. There exists two types of blocking of SH groups, irreversible and reversible blocking. Reversible blocking of SH-groups is mediated by an S-S (disulfide) exchange reaction. If the functional SH group in TGase was blocked by SH-containing compounds using S-S bond formation, the catalytic activity would be lost and activity would be recovered upon removal of the compound by adding high concentration of SH-containing reagents. The particular SH-containing reagent tested was 2-mercaptoethanol, a well known SH reagent. The addition of 2-mercapto-ethanol alone did not affect the cross-linking reaction (Fig. 4). Ground garlic (5%) alone strongly suppressed the reaction. However, cross-linking of myosin HC was observed when garlic was added together with 2-mercaptoethanol. A 2-mercaptoethanol concentration around 200 mM was required to remove the inhibition completely when the amount of garlic was 5%. There were possible two explanations for these observations. The first one was that 2-mercaptoethanol reacted with the inhibitory components in garlic, which the interfered with the putative inhibitory compound's ability to react with the SH group of TGase. Another explanation is that 2-mercaptoethanol expelled the bound component from SH group of TGase resulting in a recovery of the enzyme activity. To distinguish between these two possibilities, we designed the following experiment. First, salted surimi was added by garlic and incubated for 30 min, and then 2-mercaptoethanol was added to the surimi. The mixture was incubated for further 2 hr to examine the recovery of the enzyme activity. Further incubation of the mixture clearly produced the cross-linked myosin HC. This result clearly indicated that 2-mercaptoethanol removed the bound compound from the enzyme. In order to confirm

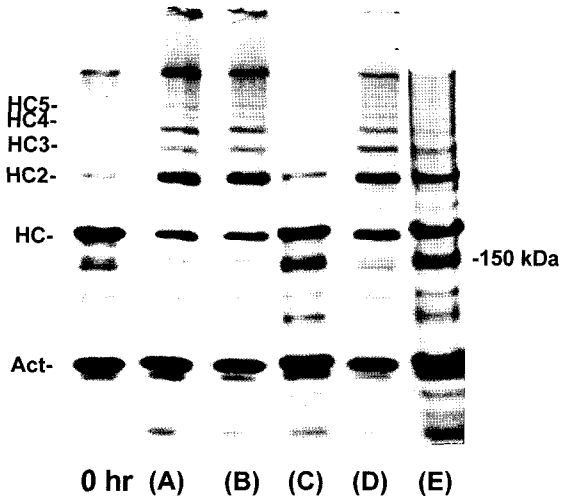


**Fig. 4. Reversible recovery of cross-linking by 2-mercapto-ethanol.** Surimi paste was incubated for 2 hr at 25°C with no additives (A), 200 mM 2-mercaptoethanol alone (B), 5% garlic alone (C), 200 mM 2-mercaptoethanol, and 5% garlic (D). 2-Mercaptoethanol was added to the surimi pre-incubated with 5% garlic for 30 min, and was further incubated for another 2 hr (E).

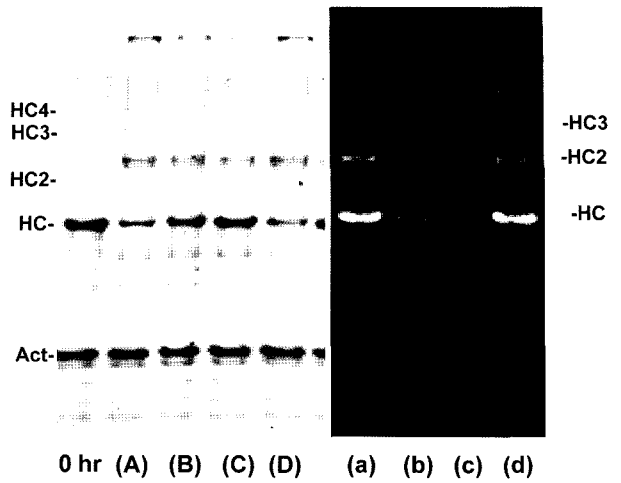


**Fig. 5. Inhibitory effect of alliin on cross-linking activity.** Surimi paste ground with 0.5 M NaCl together with no additives (A, a), 3% garlic (B, b), 3% garlic and 1.5% alliin (C, c), 5% alliin (D, d), were incubated at 25°C in the presence of 2 mM MDC. Lanes (A)-(D) are visualized by Coomassie Blue staining and lanes (a)-(d) are visualized using MDC fluorescence.

that TGase activity was reversibly recovered by 2-mercaptoethanol, recovered incorporation of MDC upon addition of 2-mercaptoethanol was investigated. The inhibition of MDC incorporation by garlic was recovered by 2-mercaptoethanol (data not shown).

**Is alliin involved in the inhibition of cross-linking?** Garlic contains various SH-containing compounds. The most abundant of these SH-containing compounds is alliin and alliin is converted to allicin by alliinase (a cystein sulfoxide lyase). It is well known that allicin is a very unstable compound that cannot be stored at room temperature. As the compounds that inhibited the TGase activity were heat stable, the inhibitory compound was unlikely to be allicin. Nevertheless, we studied whether the addition of the alliin causes the inhibition of HC cross-linking (Fig. 5). Commercial alliin directly added to surimi preparations did not affect the MDC incorporation. To test the possibility that compounds derived from alliin show the inhibition, alliin was added together with ground garlic. Garlic content was reduced to 3%. As the garlic content was reduced, we observed a slight, but visually detectible incorporation of MDC into HC. When alliin was added to surimi preparations together with the ground garlic, MDC was no longer incorporated with myosin HC, suggesting that inhibition by garlic was increased by alliin. Therefore, it appears that alliin was converted into inhibitory compounds by some enzymes contained in garlic.

**Does garlic universally interfere with TGase activity?** As the inhibitory compound in garlic was hypothesized to bind to SH groups of TGase, we investigated the possibility that the activity of other TGases such as microbial TGase (MTGase) might be inhibited by the putative inhibitory compound contained in garlic. In this experiment, the activity of TGase should be inhibited by adding ethylenediaminetetraacetic acid (EDTA) because its activity is calcium ion dependent. Myosin HC cross-linking was suppressed by adding EDTA, although the degraded fragments were detected (Fig. 6). The cross-linking reaction was also inhibited by addition of garlic alone where the protein band pattern was similar to that elicited by the addition of EDTA. Addition of MTGase to the surimi preparation containing EDTA produced cross-linked products. The 'recovery' of cross-linking occurred because the MTGase does not require calcium ion for the

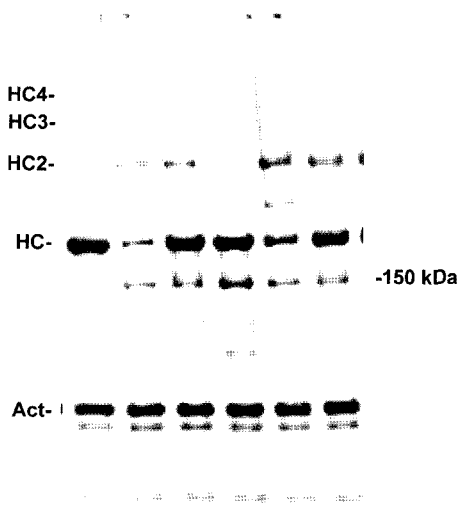


**Fig. 6. Inhibition of MTGase activity by garlic.** Surimi paste ground with 0.5 M NaCl together with no additives (A), 5 mM EDTA (B), 5% garlic (C), 5 mM EDTA and 5 unit/g MTGase (D), 5 mM EDTA, 5 unit/g MTGase, and 5% garlic (E) were incubated at 25°C for 2 hr.

activation. Ground garlic was then added to the surimi preparation containing EDTA and MTGase. The cross-linking reaction was then significantly suppressed by garlic. We therefore conclude that SH-containing compounds in garlic strongly inhibit TGase activity irrespective of the origin of the enzyme (i.e., bacterial versus fish).

**Isolation and characterization of the putative inhibitory compound from garlic** Much effort was devoted to improvement of the gel properties of thermal gels such as surimi. One of the fundamental challenges was to introduce setting or pre-heating process at relatively low temperatures. The pre-heating process allows TGase to produce myosin cross-linked polymers. The products (cross-inked polymers) are believed to be closely related to the improved gel strength.

In this study, we showed that garlic contains a very strong inhibitor of the enzyme that catalyzes myosin HC cross-linking. We attempted to isolate and identify the putative inhibitory compound(s). The outer solution obtained by the dialysis of ground garlic was concentrated by lyophilization and dissolved in water. This solution was applied to a SepPak C18 (residue volume of 12 mL), and roughly fractionated by increasing methanol concentration. As the inhibitory compounds were hypothesized to be water soluble, we expected to demonstrate the strong inhibition of cross-linking activity by the fraction eluted with water. However, the fraction only weakly inhibited the cross-linking reaction. All fractions eluted with various methanol concentrations showed no clear inhibition of cross-linking activity. Nuclear magnetic resonance analysis of the water soluble fraction showed that major components in the water soluble fraction are sugars. We did not attempt a further identification of the compounds.

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