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Plant proteases and anti-bacterial substances in *Allium sativum* L. varieties

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Abstract:
Allium sativum L. protease still remains largely understudied although new varieties of garlic appear quite often, e.g., *lanang* garlic. This study tested the antibacterial effect of garlic and the effectiveness of various *A. sativum* proteases as meat tenderizers. The research involved powder extracts of four varieties of *A. sativum*: *kating*, *lanang*, black garlic, and *sin-chung*. The degradation kinetics was defined based on the Lineweaver-Burk equation. The degradation zones were measured using sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). Scan electron microscopy served to test the changes in meat connective tissue.
Lanang demonstrated the largest inhibition zones against *Escherichia coli* (9.75 ± 0.15 mm) and *Staphylococcus aureus* (1.04 mm). *Sin-chung* protease degraded beef protein with the highest V_{max} of 0.1818 μg/μL/min at 10–22 KDa (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin), and 100–140 KDa (protein C). The same garlic variety degraded mutton meat protein at 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin) with V_{max} of 0.1135 μg/μL/min.
All four *A. sativum* proteases proved to be quite effective meat tenderizers.

Keywords: *Allium sativum* protease, *lanang* garlic, *kating* garlic, black garlic, *sin-chung* garlic

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INTRODUCTION

The benefits of garlic (*Allium sativum* L.) remain a relevant scientific issue, even though some of them have become a long-established concept. Almost all researchers agree on the role of *A. sativum* as a source of prose inhibitors and antibiotics [1–11]. Garlic is known for its antifungal, antibacterial, hypolipidemic, anti-atherosclerosis, and anticarcinogenic properties, not to mention that garlic is a world-famous culinary spice [8, 12–19].

This research aimed at identifying the connection between the *A. sativum* protease inhibitor and various scientifically-established concepts regarding the role of protease in *A. sativum* plants. Proteases are important for the metabolic processes of plant cell growth [20–22]. Researchers have reached no consensus about the relationship between *A. sativum* protease and its inhibitors. The role of *A. sativum* protease remains quite vague because its effectiveness is often disguised by the functions of other spices [23, 24]. For instance, the effect of *A. sativum* protease as a meat tenderizer cannot be separated from other ingredients in the marinating process [23, 25]. Figure 1 proves that very few studies report the role of *A. sativum* protease as a meat tenderizer. We collected around 750 papers published in 2010–2023 that featured *A. sativum* and searched for *A. sativum* protease only to find some 82 results (0.1%). Thus, the poor scientific coverage of *A. sativum* protease and its properties became the background for this research.

In Indonesia, *A. sativum* is represented by such varieties as black garlic, *kating*, and *sin-chung*. *Lanang* garlic, or single garlic, is an accidental new variety that appeared as a result of unsuitable planting environment in

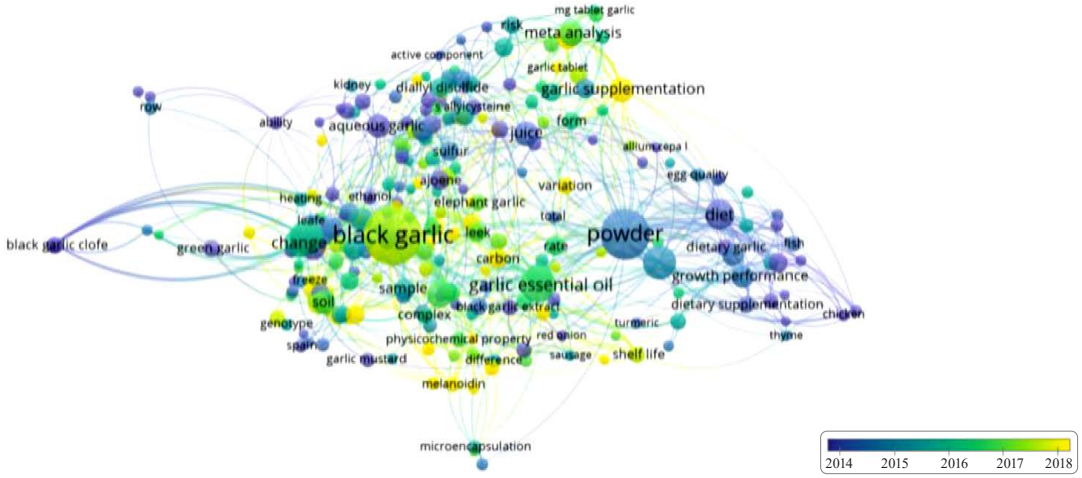


Figure 1 *Allium sativum* L. in scientific publications

the Sarangan area, Magetan, East Java. *Lanang* is more of a medicinal plant than a culinary spice. Poor shoot growth in the canopy inhibits the clove budding, which results in a single big garlic clove. Such growth is suspected to be due to the effect of plant protease on the formation of plant cells [22]. In this regard, the new variety represents a prospective material for protease studies. We compared *lanang* garlic and its properties with other three varieties of *A. sativum* by testing their effect on the inhibition of Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) bacteria.

The research objectives included the following tasks:
– to test the inhibitory power of Gram-positive and Gram-negative bacteria of the four varieties of *A. sativum*; and
– to compare the effectiveness of *A. sativum* protease in four garlic varieties.

We measured the effectiveness based on the following parameters:

1. Degradation kinetics. This parameter included such measurements as the maximal speed (V_{max}) of protein degradation in beef and mutton substrates and the Michaelis Menten constant (K_M);

2. Meat protein degradation zone. It involved sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE); and

3. Effect of *A. sativum* protease on muscle connective tissue, perimysium, endomysium, and collagen in mutton and beef using scan electron microscopy.

STUDY OBJECTS AND METHODS

We purchased *lanang*, *kating*, black garlic, *sin-chung*, beef, and mutton at a local supermarket. The pure bacterial cultures of *Eschericia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were obtained from Merck KGaA, Darmstadt, Germany. The control samples were beef and mutton without *Allium sativum* L., while experimental samples included beef and mutton meat with different varieties of *A. sativum*, namely *lanang*, *kating*, black garlic, *sin-chung*.

Sample treatment. To powder the garlic samples, we peeled the garlic skin and cut each clove into three pieces. After that, we baked it in an oven at 70°C for 72 h. After cooling it at room temperature (25°C) for 15 min, we pulverized the mass with a blender and sieved it. Finally, we re-ovened the powder at 70°C for 24 h.

We extracted 100 g of dried garlic powder using water (100 mL) for 72 h, then filtered and evaporated it in an evaporator for 1 h to obtain a thick extract. The final extract concentration used for analysis was 20% (20 mg/mL).

The beef and mutton were sliced into thin slices (4 cm×4 cm×2 mm). The meat was smeared with 20% garlic extract as the weight of the garlic extract to the weight of the meat. After letting it rest for 30 min, we stored the meat for 60 min at 30–35°C to prevent thermal changes. Untreated meat served as control. The protein degradation kinetics analysis (SDS PAGE) took place at 30 and 60 min, but the scan electron microscopy test was performed at 60 min.

Bacterial inhibition test. After extracting 100 g of the dry garlic powder for 72 h, we filtered and evaporated it in an evaporator for 1 h. The procedure was followed by the water bath. The resulting thick extract had a concentration of 20% (20 mg/mL) each.

The strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were taken at 200 µL and spread out with a sterile spreader glass in a petri dish containing Muller Hinton Agar and Mannitol Salt Agar. Disk blanks were impregnated with 15 µL of stock extract. One disk served as a negative control whereas the other disc was filled with sample extracts. After incubating them at 37°C for 24 h, we observed the diameter of the inhibition zone in line with the procedure we described in [26].

Protein content. We used the biuret method with a UV-Vis spectrophotometer at λ = 595 nm to determine the protein content in the meat samples [27].

Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). We performed the analysis of protein degradation using SDS-PAGE in line with the

procedure specified by the Association of Official Analytical Chemists [28]. The analysis involved acrylamide gel electrophoresis: top (5% stacking gel) and bottom (12% separating gel).

Scanning electron microscopy. This procedure relied on Běhalová *et al.* [29]. The meat structure was analyzed using a scanning electron microscope (ZEISS, type EVOMA 10). The image was displayed using a secondary electron (SE) detector.

Maximal speed (V_{max}) and Michaelis Menten constant (K_M). The kinetics of protein degradation involved the Lineweaver-Burk equation, which is the inverse of the Michaelis Menten equation [30]. The relationship between reaction rate V and substrate concentration S changed to $1/V$ and $1/S$:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{S} + \frac{1}{V_{max}} \quad (1)$$

where $1/V$ is the y-axis and $1/S$ is the x-axis; $y = bx + a$; $V_{max} = 1/a$; $K_m = V_{max} \times b$.

RESULTS AND DISCUSSION

Bacterial inhibition. *Lanang* garlic had the greatest bacterial inhibition among all *Allium sativum* L. varieties, both in relation to Gram-negative and Gram-positive bacteria. The inhibition power for Gram-negative *Eschericia coli* was 9.75 ± 0.15 mm. For Gram-positive *Staphylococcus aureus*, the inhibition zone was 1.04 mm.

Kating garlic was able to inhibit *E. coli* with an inhibition zone of 7.54 ± 0.25 mm. For *S. aureus*, it did not exceed 1 mm. Black garlic and *sin-chung* garlic extracts yielded no inhibition results.

Table 1 sums up the results for all the *A. sativum* varieties in this research.

Lanang demonstrated the best inhibition results for Gram-negative and Gram-positive bacteria. It might owe its effectiveness to homogenate allicin (S-(2-propenyl)2-propene-1-sulfinothionate), which is known for its antibacterial activity [31]. *Kating* also inhibited *E. coli* and *S. aureus*, but to a much lesser extent. Black garlic and *sin-chung* produced no inhibition effect at a concentration of 20 mg/mL. Probably, the process of forming active bacterial substances in the cell enlargement process and the growth area were far from optimal [32].

Kinetics of meat protein degradation. The control samples of beef and mutton, which were untreated with garlic extracts, showed no significant difference ($p > 0.05$). The mutton samples treated with extracts of *kating*, *lanang*, black garlic, and *sin-chung*, on the contrary, demonstrated significant differences ($p < 0.05$) after 30 and 60 min of processing. The same was true for the experimental beef ($p < 0.05$).

The *sin-chung* garlic extract was able to reduce the protein content, followed by black garlic and *kating*. *Lanang*, however, showed little effect on the degradation of meat protein. Table 2 illustrates the changes in protein levels for the mutton and beef samples.

Protein degradation kinetics was analyzed using the Lineweaver-Burk equation. Figure 2 shows the relationship of velocity ($1/V$) to substrate degradation ($1/S$). Figure 3 illustrates the changes in the concentration of protein substrate (S).

Table 1 *Allium sativum* L. varieties: bacterial inhibition effect

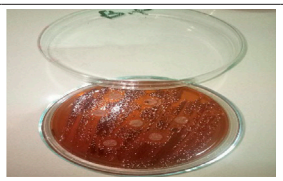
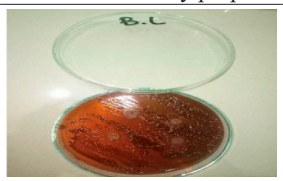
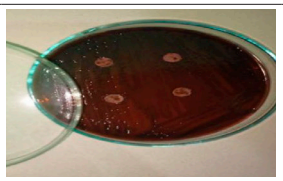
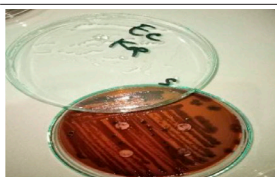
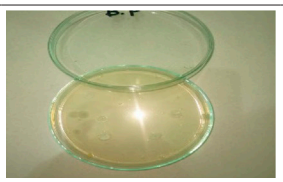
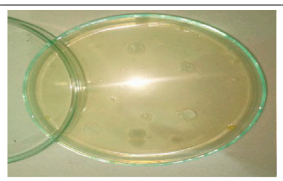
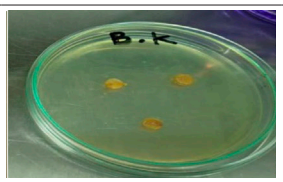
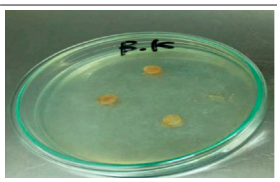
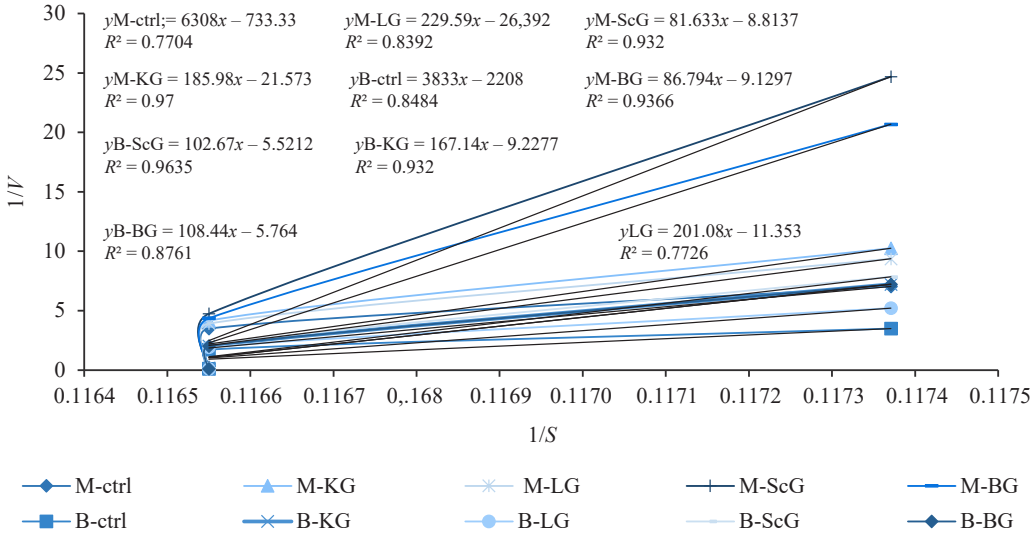
	Kating	*Lanang*	Black garlic	*Sin-chung*
Appearance				
Characteristics	Small wrinkled cloves clustered together	Single cloves; the smallest size among other varieties	Clustered black cloves	Large clustered cloves
		Inhibitory properties		
Escherichia coli				
	Diameter of inhibition zone: 7.54 ± 0.25 mm	Diameter of inhibition zone: 9.75 ± 0.15 mm	No zone of inhibition detected	No zone of inhibition detected
Staphylococcus aureus				
	Diameter of inhibition zone: < 1 mm	Diameter of inhibition zone: 1.04 mm	No zone of inhibition detected	No zone of inhibition detected

Table 2 Protein contents, μg/μL in meat samples

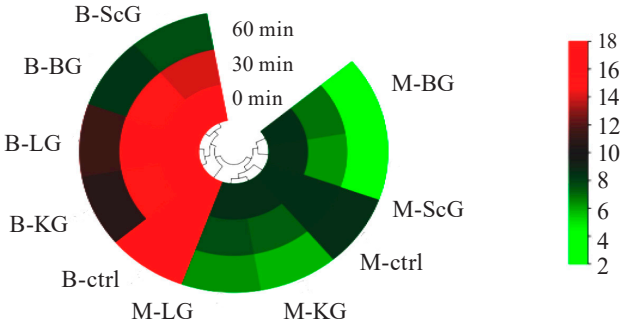
Sample	0 min	30 min	60 min
Mutton, control	9.39 ± 0.15[a]	9.41 ± 0.05[a]	9.52 ± 0.12[a]
Mutton + *kating*	8.38 ± 0.13[c]	7.32 ± 0.11[b]	5.78 ± 0.06[a]
Mutton + *lanang*	8.52 ± 0.15[c]	7.60 ± 0.05[b]	6.30 ± 0.08[a]
Mutton + black garlic	8.50 ± 0.18[c]	6.90 ± 0.04[b]	2.90 ± 0.05[a]
Mutton + *sin-chung*	8.47 ± 0.11[c]	6.29 ± 0.15[b]	2.34 ± 0.18[a]
Beef, control	18.35 ± 0.25[a]	18.41 ± 0.15[a]	18.31 ± 0.35[a]
Beef + *kating*	17.35 ± 0.25[c]	15.17 ± 0.35[b]	10.77 ± 0.13[a]
Beef + *lanang*	17.35 ± 0.15[c]	15.47 ± 0.15[b]	11.40 ± 0.15[a]
Beef + black garlic	17.35 ± 0.15[a]	14.70 ± 0.35[b]	8.23 ± 0.18[a]
Beef + *sin-chung*	17.32 ± 0.14[c]	14.10 ± 0.05[b]	7.60 ± 0.17[a]

Anova Tukey HSD post-hoc test was performed at standard $p < 0.05$. The same superscripts indicate no significant difference between the samples. Mutton and beef samples smeared with extracts of *kating*, *lanang*, black garlic, and *sin-chung* were compared with the untreated control



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic

Figure 2 Kinetics of protein degradation per 1 min in beef and mutton samples



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic

Figure 3 Changes in protein content per 1 min in beef and mutton samples

In the mutton samples, *sin-chung* protease had the highest V_{max} in the protein degradation process of 0.1135 μg/μL/min using a substrate of 9.19 μg/μL. The *lanang* protease showed the lowest V_{max}, namely 0.0378 μg/μL/min. The results for black garlic and *kating* were 0.1096 and 0.0464 μg/μL/min, respectively. The *sin-chung* protease had the best degrading results for beef protein with V_{max} of 0.1818 μg/μL/min and substrates ranging from 18.54 μg/μL. The black garlic protease produced V_{max} of 0.1735 μg/μL/min while *kating* protease was 0.1078 μg/μL/min. The *lanang* protease had the lowest effect: 0.088 μg/μL/min (Table 3).

The process of forming antibacterial active substances does not always coincide with the formation of plant cells during cell formation/growth process, which involves plant proteases. This research succeeded in proving that black garlic and *sin-chung* were in that phase. In both cases, protease tenderized the meat. The *sin-chung* variety had the highest protein degradation V_{max} in mutton (0.1135 μg/μL/min) and beef (0.1818 μg/μL/min). Black garlic protease had the second-best result, followed by *kating* and *lanang*. The obtained results confirm those reported by Bar *et al.* regarding the formation of anti-bacterial substances and Sharma & Gayen regarding the growth process of *A. sativum* [22, 32].

Meat protein degradation zone. To measure the protein degradation zone, we smeared the mutton and beef

Table 3 Kinetics of beef and mutton protein degradation under the effect of *Allium sativum* L. protease enzyme

Sample	V_{max}, µg/µL/min	K_M, µg/µL
Mutton, control	0.001363698	8.602209
Mutton + *sin-chung*	0.113459727	9.190238
Mutton + *lanang*	0.037878788	8.674242
Mutton + black garlic	0.109649123	9.429825
Mutton + *kating*	0.046360686	8.576727
Beef, control	0.004545455	17.42273
Beef + *sin-chung*	0.181818182	18.54545
Beef + *kating*	0.107793468	18.01660
Beef + black garlic	0.173490632	18.81332
Beef + *lanang*	0.088082445	17.70457

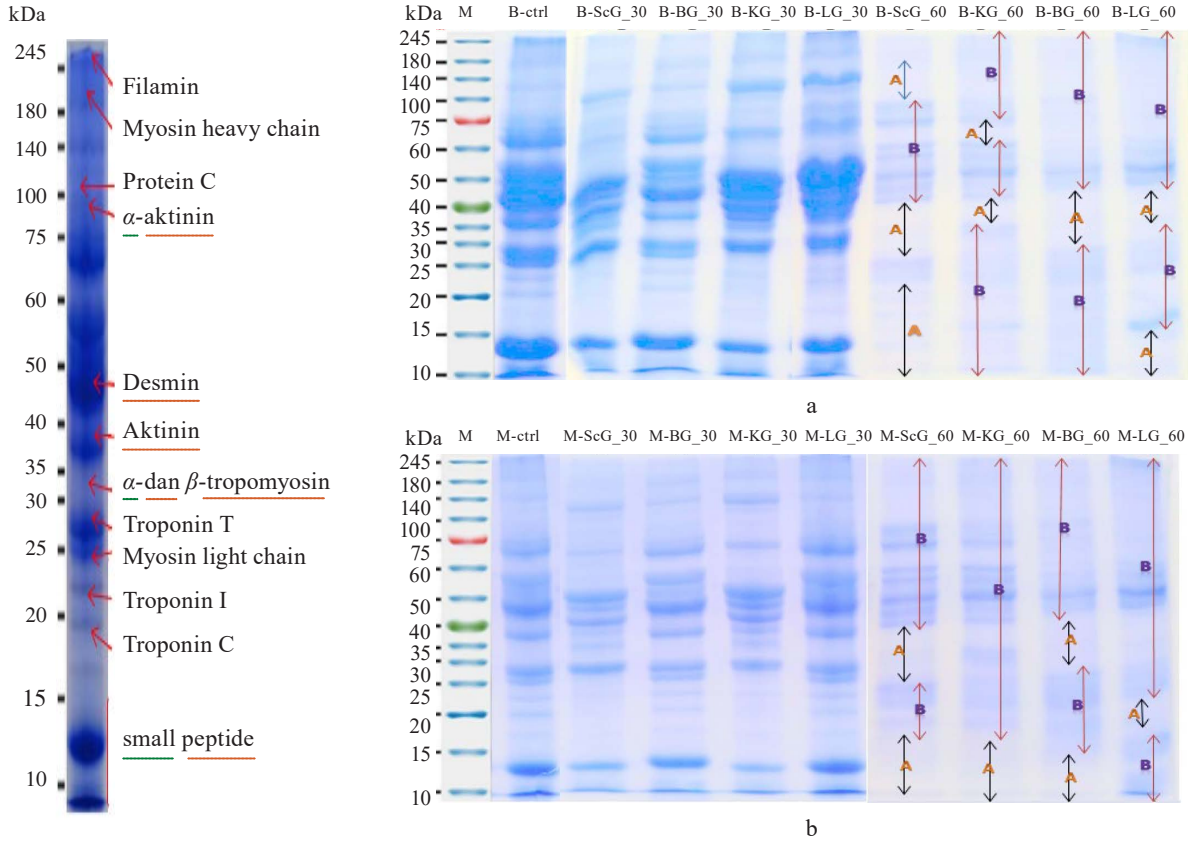
samples with extracts of *kating*, *lanang*, black garlic, and *sin-chung* at 30 and 60 min and compared the obtained results with those for the untreated control samples.

In beef (Fig. 4a), the garlic extracts produced no mild degradation within the first 30 min. Mild degradation is marked by a change in color from blue to purple while complete degradation means a loss of color. Both were clearly visible after 60 min. The *sin-chung* protease demonstrated complete degradation in the area of 10–22 KDa (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin), and 100–140 KDa (Protein C). Other areas showed mild degradation only.

The *kating* protease was able to degrade proteins in the 35–40 KDa (actin) and 60–62 KDa zones. Other areas were only slightly degraded. The black garlic protease produced a degradation effect in the 30–42 KDa zone (*α*- and *β*-tropomyosin, actin). Meanwhile, *lanang* had the smallest degradation area of 10–17 KDa (small peptide) and 40–50 KDa (desmin).

In mutton (Fig. 4b), the proteases produced no degradation effect within the first 30 min. For all garlic varieties, degradation started at 60 min. *Lanang* was able to degrade only 20–23 KDa (troponin I). S*in-chung* had a degradation zone at 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin). The back garlic protease was in the 10–15 KDa and 35–40 KDa ranges. *Kating* was effective in the 10–17 KDa range.

Therefore, all four *A. sativum* proteases proved to be effective meat tenderizers. For beef, the *sin-chung* extract succeeded in degrading proteins in a fairly wide area of 10–22 KDa (small peptide, troponin C, troponin I), 25–40 KDa (myosin light chain, troponin T, *α*- and



M – mutton; B – beef; ctrl. – control; KG – *kating* garlic; ScG – *sin-chung* garlic; BG – black garlic; LG – *lanang* garlic; A – complete degradation; B – mild degradation; KDa – protein molecular weight (Kilo Dalton); M – markers.

Example of reading code: B-BG_30 stands for beef + black garlic for 30 min; M-BG_60 stands for mutton + black garlic for 60 min. Other codes follow the same pattern.

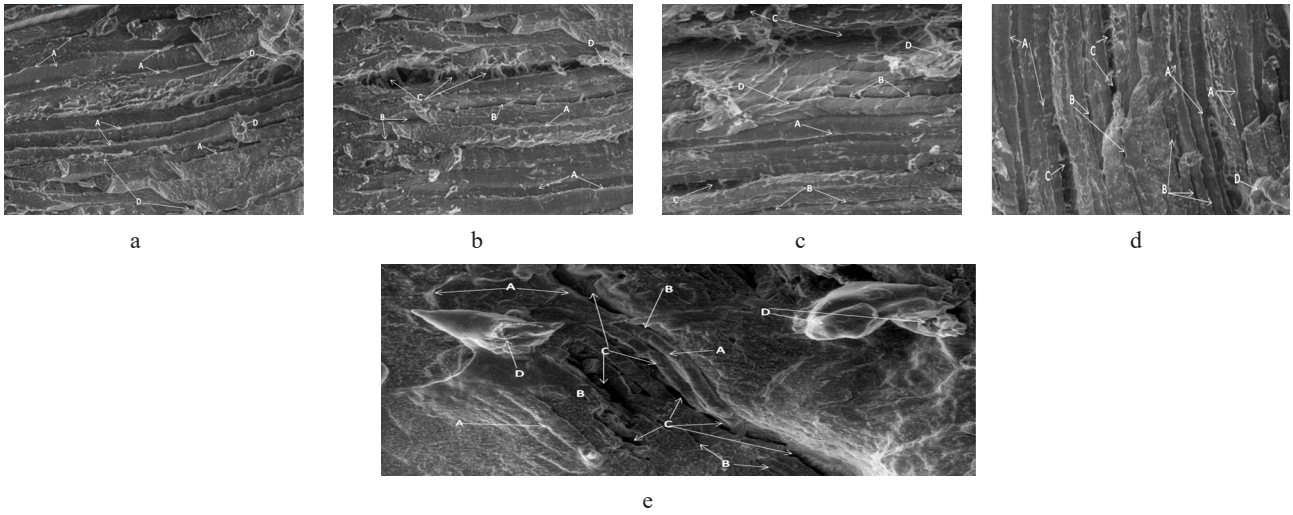
Figure 4 Complete and mild degradation zones for beef (a) and mutton (b): SDS-PAGE

β-tropomyosin, actin), and 100–140 KDa (protein C). As for the mutton samples, the *sin-chung* extract managed to degrade only 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, *α*- and *β*-tropomyosin, actin). Meanwhile, *lanang* had the smallest degradation zone: 10–17 KDa (small peptide) and 40–50 KDa (desmin) for beef and 20–23 KDa (troponin I) for mutton.

Effect of *A. sativum* protease on meat connective tissue. Collagen is the most abundant component in muscle tissue, where it forms perimysium and endomysium. Perimysium separates muscle fibers while endomysium coats them. Perimysium and endomysium release muscle tissue in the form of tears or cracks.

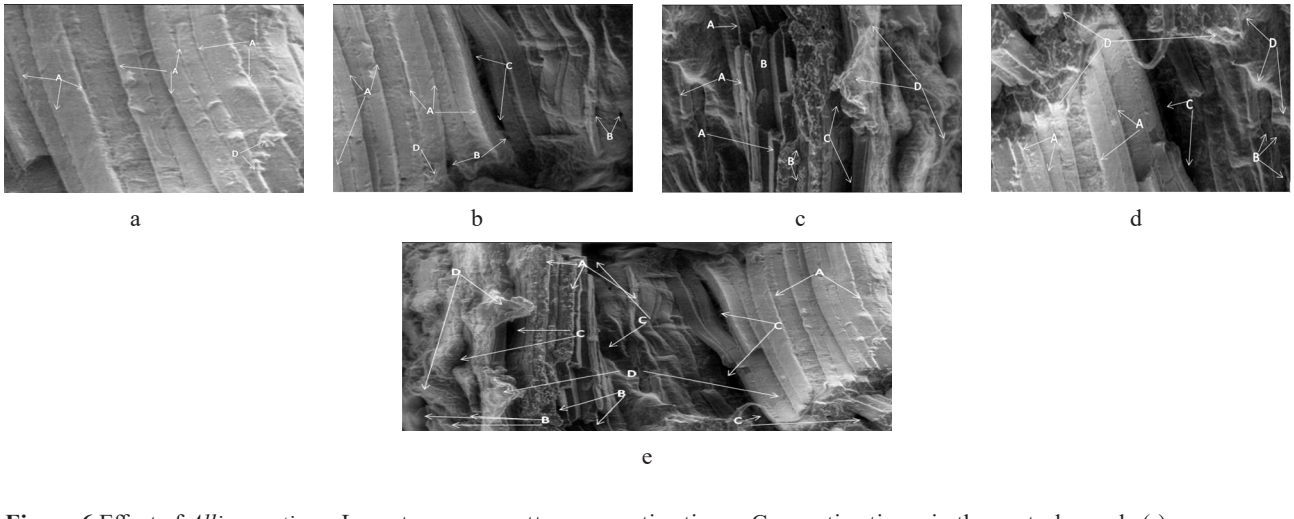
Figure 5 illustrates the effect of *A. sativum* protease on beef connective tissue. In the control meat (Fig. 5a), muscle tissue remained tight, and collagen dominated. In the sample treated with black garlic (Fig. 5b), endomysium connective tissue looked elongated and wide, with perimysium predominating. Some strong muscle tissue remained, and collagen was no longer predominant. The *lanang* protease (Fig. 5c) affected endomysium and perimysium at several points. Strong-bound muscle tissue predominated, but collagen was reduced. *Kating* (Fig. 5d) resulted in dominant perimysium. Small endomysium tissue was visible in some areas, and strong muscle tissue was detected in all directions. *Sin-chung* (Fig. 5e) was able to change the dominance of collagen and muscle tissue, and they became less dominant. Large endomysium predominated at several points, although perimysium was visible only on one side.

Figure 6 illustrates the effect of *A. sativum* protease on connective mutton tissue. The control sample (Fig. 6a) was predominated by strong muscle tissue and collagen. No visible loss of muscle tissue was detected:



a b c d e

Figure 5 Effect of *Allium sativum* L. proteases on beef connective tissue. Connective tissue in the control sample (a) was degraded by black garlic (b), *lanang* (c), *kating* (d), and *sin-chung* (e), which affected muscle tissue (A), perimysium (B), endomysium (C), and collagen (D)



a b c d e

Figure 6 Effect of *Allium sativum* L. proteases on mutton connective tissue. Connective tissue in the control sample (a) was degraded by black garlic (b), *lanang* (c), *kating* (d), and *sin-chung* (e), which affected muscle tissue (A), perimysium (B), endomysium (C), and collagen (D)

perimysium and endomysium did not appear under these conditions. Black garlic (Fig. 6b) resulted in a long and wide endomysium network, though at one point only. Perimysium appeared at several points. Muscle tissue predominated but collagen was no longer visible. *Lanang* (Fig. 6c) had very little effect on perimysium and endomysium formation. Tightly bound muscle tissue and collagen still predominated on all sides of the meat sample. The *kating* protease (Fig. 6d) produced large endomysium and small clustered perimysium visible on a few sides. Muscle tissue and collagen still predominated on all sides of the meat sample. *Sin-chung* (Fig. 6e) had a very different effect. Large and long endomysium dominated on all sides of the meat sample. Perimysium demonstrated a similar picture but was scattered at several points. In this sample, collagen and muscle tissue were only seen in one order and were clustered together.

All four *A. sativum* proteases were able to separate myofibers from the perimysium, which is the most vulnerable tissue. This experiment was able to catalyze the effect of perimysium as it separated muscle fibers in muscle connective tissue. Perimysium is a fascicle that can be classified into primary, secondary, and tertiary fascicles, based on the diameter [33].

Sin-chung and black garlic had a prominent effect on the formation of dominant endomysium, which is the first step in meat tenderizing. Probably, when endomysium detached from sarcomere, it surrounded the muscle fibers of basal lamina, proteoglycans, collagen, and lamina. As a result, the endomysium formation left tears or cracks on the meat surface. The obtained results were consistent with those reported by Swasdison & Mayne regarding endomysium formation [34]. The control samples (Figs. 5a and 6a) revealed no endomysium and perimysium tissue because the cross-linked tissue was still strong in muscle tissue and collagen.

CONCLUSION

In this research, the *lanang* garlic variety demonstrated the greatest antibacterial properties: its inhibition zone was 9.75 ± 0.15 mm against *Escherichia coli* and 1.04 mm against *Staphylococcus aureus*. Black garlic and *sin-chung* demonstrated no inhibitory power, probably, because the process of forming anti-bacterial substances does not always coincide with the process of plant growth, which involves plant proteases.

All four *Allium sativum* L. proteases proved to be effective meat tenderizers. The *sin-chung* extract possessed the most effective plant protease in this process. Its protease was able to degrade beef protein with the highest V_{max} of 0.1818 μg/μL/min in the 10–22 KDa range (small peptide, troponin C, and troponin I), 25–40 KDa (myosin light chain, troponin T, α- and β-tropomyosin, actin), and 100–140 KDa (protein C). In mutton, it was effective only in the 10–17 KDa (small peptide) and 25–40 KDa (myosin light chain, troponin T, α- and β-tropomyosin, actin) ranges with V_{max} of 0.1135 μg/μL/min. The *lanang* protease showed the weakest protease enzyme activity: a small degradation zone in the area of 10–17 KDa (small peptide) and 40–50 KDa (desmin) with V_{max} of 0.0881 μg/μL/min for beef. For the mutton samples, its result was 20–23 KDa.

CONTRIBUTION

All the authors were equally involved in the research analysis and manuscript writing.

CONFLICT OF INTEREST

The authors declare no conflict of interests regarding the publication of this article.

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