EFFECTS OF COLLAGENASE FROM *Shewanella* sp. ON MEAT TENDERIZATION

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Abstract – Effects of a new collagenase, from *Shewanella* sp. strain C35 (CL-C35), were investigated in an effort to develop a new enzyme for increased meat tenderness, without negatively affecting meat texture. Meat toughness is associated with intramuscular collagen, which is the main component of connective tissue. Due to broad substrate specificity, the enzymes currently used to tenderize meat degrade not only collagen but also myofibrillar proteins, resulting in unfavorable meat texture. Moreover, these enzymes don't act at meat strage temperatures. Compared to commercially used tenderizers, papain and collagenase, CL-C35 showed high selectivity for acid-soluble collagen in Tris-HCl buffer (pH 7.4). Additionally, CL-C35 had strong collagenolytic activity at 4°C, and was easily inactivated by heating. CL-C35 treatment at 4°C reduced the shear force value of meat, and differences were observed in the disruption pattern of the connective tissue ultrastructure under scanning electron microscopy. These results suggest that the cold-adapted, collagen-specific enzyme CL-C35 holds promise for use as a meat tenderizer.

Key Words – Connective tissue, Meat tenderizer, Myofibrillar protein

I. INTRODUCTION

Tenderness is one of the most important factors consumers use in judging meat sensory quality. Meat toughness can be loosely divided into actomyosin toughness, attributed to myofibrillar proteins, and background toughness due to the presence of connective tissue [1]. While the structural features of myofibrillar proteins are altered with aging, those of connective tissue remain largely unchanged [2]. Specifically, intramuscular collagen, which is the main protein component of connective tissue, forms a firm network structure, and is a major factor in meat toughness.

Many chemical and physical methods have been developed for improving meat tenderness. Enzymatic treatment is a widely used technique for meat tenderization. At present, tenderizing enzymes, such as bromelain, ficin and papain, are commonly used. However, these enzymes have very broad specificity and can, therefore, indiscriminately break down major muscle proteins (connective tissue/collagen and myofibrillar proteins), resulting in the 'mushy/grainy' texture associated with over-tenderized meat [3]. Furthermore, these proteases are active at high temperatures; i.e., papain has an optimum temperature of 80°C, and exhibits approx. 10% activity at refrigeration temperatures. In addition, papain is heat-stable and is, therefore, not readily inactivated, resulting in over-tenderization even after cooking [4].

Consequently, the ideal enzymatic meat tenderizer would 1) be specific for collagen in the connective tissue, 2) exhibit collagenolytic activity at relatively low temperatures, and 3) be susceptible to heat denaturation.

Ogawa et al. isolated *Shewanella* sp. strain C35 from soil [5]. This microorganism is a rod-shaped, Gram-negative, psychrophilic bacterium that produces a collagenolytic enzyme. This enzyme exhibits stronger substrate specificity for collagen compared to commercial enzymes such as papain and *Clostridium histolyticum*-derived collagenase, and is active at low temperatures in Tris-HCl buffer. These characteristics indicate that CL-C35 is a promising enzyme for meat tenderizaitoin.

In the present study, we investigated the effects of CL-C35 on meat tenderization in comparison to commercially available enzymes.

II. MATERIALS AND METHODS

Materials

Papain and collagenase (CL-AS) were purchased from Amano Enzyme Inc. of Japan. The outside flat of beef was obtained from Itoham Foods Inc. of Japan.

Collagen and Actomyosin extraction

Collagen was extracted from porcine skins according to the procedure described by Ogawa et al. [6]. Actomyosin was prepared from pork *longissimus dorsi* muscle according to the slightly modified method of Szent-Györgyi [7].

Enzyme preparation

Shewanella sp. strain C35, isolated from soil, was aerobically cultured for 7 days using a jar fermenter (MBF, EYELA) under the following conditions: incubation temperature, 6°C; 15% dissolved oxygen; M1 synthetic medium adjusted to pH 7.4 using 0.1 M HCl [8]. The culture medium was centrifuged at 10,000 × *g* for 10 min, and the supernatant was diluted with 5 volumes of 10 mM Tris-HCl buffer (pH 7.8) containing 10 mM $CaCl_2$, and chromatographed on a DEAE-Sepharose Fast Flow column (50 × 100 mm) at a flow rate of 1 ml/min.

Enzyme Assay

For the substrates, 8 mg/ml collagen solution in acetic acid (pH 4.0) and 8 mg/ml actomyosin solution in 0.6 M NaCl were prepared. The enzymes, CL-C35, papain or CL-AS, were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$. Each reaction mixture was incubated at 4-100°C. Distilled water was used in place of the enzyme for the control. The reaction was stopped by the addition of SDS sample buffer and heating for 3 min at 100°C. One unit of collagenase or protease activity was defined as the amount of enzyme required to decrease the area of collagen β-chain or myosin heavy chain by 1% in 1 min on SDS-PAGE.

Reaction mixture

10 mM Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$	40 µl
substrate	10 µl
Enzyme	10 µl

Texture measurement

To investigate the effects of meat tenderization, outside flat of beef and Japanese Wagyu beef were used as the tough meat sample and the tender meat sample, respectively. Meat was cut into small pieces (10 × 10 × 30 mm) and incubated in 2 volumes of a solution containing papain, CL-AS or CL-C35 at 4°C for 5 days. The enzymes were dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$, and the enzyme usage was 200 U/ml of collagenolytic activity at 30°C. Meat samples incubated in identical buffer at 4°C for 5 days were used as the control. After incubation, each meat sample was vacuum-sealed in a polyethylene bag and immediately heated at 60°C for 30 min in a water bath. Using a rheometer (RE2-33005B, YAMADEN), shear force value of the raw and heated meats was measured.

Scanning electron microscopy (SEM)

After enzyme treatment at 4°C for 5 days, the meat was fixed in glutaraldehyde. Samples were rinsed in distilled water and immersed in 10% NaOH, which was changed every 2 days. Then, samples were placed in 1% tannic acid, and post-fixed in 1% OsO_4. After dehydration through a graded ethanol series, the specimens were freeze-dried in t-butyl alcohol. The dried specimens were coated with osmium (OCP60A, Filgen), and observed using scanning electron microscopy (JSM-6360A, JEOL).

III. RESULTS AND DISCUSSION

Substrate specificity

All enzymes degraded collagen β-chains at 30°C (Fig. 1-A). CL-C35 slightly degraded myosin heavy chain of actomyosin, whereas papain and CL-AS treatment resulted in marked degradation (Fig. 1-B). This suggests that CL-C35 was able to selectively degrade collagen.

Temperature stability

The characteristics of each enzyme, such as optimum temperature, activity at refrigeration temperature (10°C) and thermal inactivation temperature are shown in Table 1. Papain, which is a thermophilic protease, has an optimum temperature of 80°C, whereas CL-AS has an optimum temperature of 30°C, equal to that of CL-

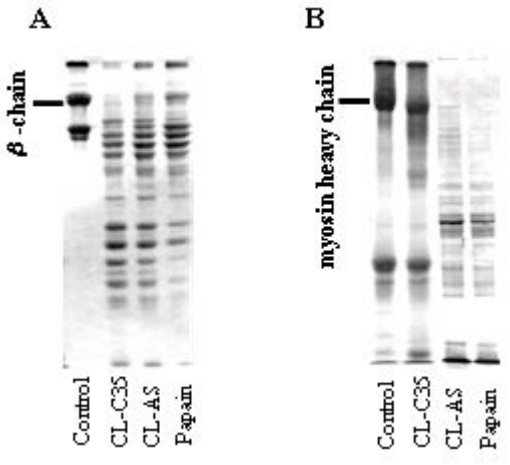

Figure 1. SDS-PAGE gel of protein degradation patterns after enzyme treatments.
(A) Collagen degradation patterns at 30°C.
(B) Actomyosin degradation patterns at 30°C.

C35. CL-C35 retained more than 50% of its activity at refrigeration temperature. However, CL-AS and papain retained only less than 15% activity at refrigeration temperature. The large decrease in enzyme activity is a negative attribute for meat tenderizers because tenderization of meat is often carried out at room temperature, prior to refrigeration. Moreover, CL-C35 rapidly loses activity at temperatures higher than 60°C, due to rapid autolysis, as compared to other enzymes, in which activity is maintained to 90°C. The thermal inactivation temperature of CL-C35 is a positive meat tenderizer attribute because it prevents over-tenderization during cooking.

Effect of meat tenderization

The results of enzyme treatment at 4°C for 5 days on meat texture are shown in Fig. 2-A. A significant decrease was observed in the shear force value of CL-C35-treated meat compared to the control, and was equivalent to that of Wagyu. On the other hand, the shear force value for papain-treated meat showed a tendency to decrease slightly, while no significant differences were observed between the control and CL-AS-treated meat. This indicates that CL-AS exhibited minimal activity at 4°C, whereas the cold-adapted CL-C35 showed an obvious meat tenderization effect.

Table 1 Characteristics of enzymes

Enzyme	CL-C35	CL-AS	Papain
Optimum temp.	30°C	30°C	80°C
Activity at 10°C	59.2%	7.4%	14.4%
Thermal inactivation temp.	60°C	90°C	90°C

Differences in shear force values of the enzyme-treated meat heated at 60°C were observed (Fig. 2-B). The shear force value of papain-treated meat was lower than that of Wagyu, whereas CL-C35-treated meat was equivalent to that of Wagyu. As for CL-AS-treated meat, a tenderization effect was not observed after heating. During heating, the tenderizing protease activity of CL-C35, which deactivated at 60°C, was stopped; however, papain-treatment resulted in over-tenderized meat, as the activity of papain is maintained at 60°C.

Scanning electron micrographs of the intramuscular connective tissue of the enzyme-treated meat are shown in Fig. 3. In the control, the collagen fibrils formed intact endomysium. CL-AS-treated meat was not significantly different from control. After CL-C35 treatment, a large amount of collagen fibril dissociation was observed, whereas papain-treated meat showed only slight fibril dissociation.

On the other hand, the myofibrillar protein disruption patterns of the enzyme-treated meat differed notably. Especially after heating at 60°C,

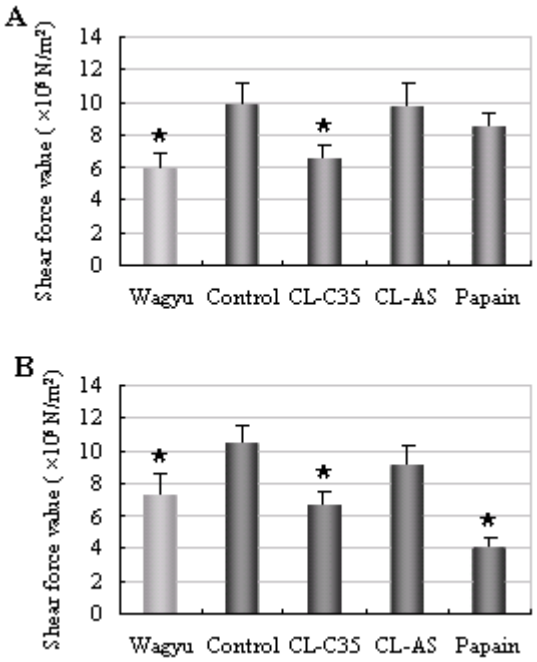

Figure 2. Shear force values of enzyme-treated meat. (A) Shear force after enzyme treatment at 4°C for 5 days. (B) Shear force after enzyme treatment at 4°C for 5 days and subsequent heat-treatment.
* Significantly different from the control at $p<0.05$

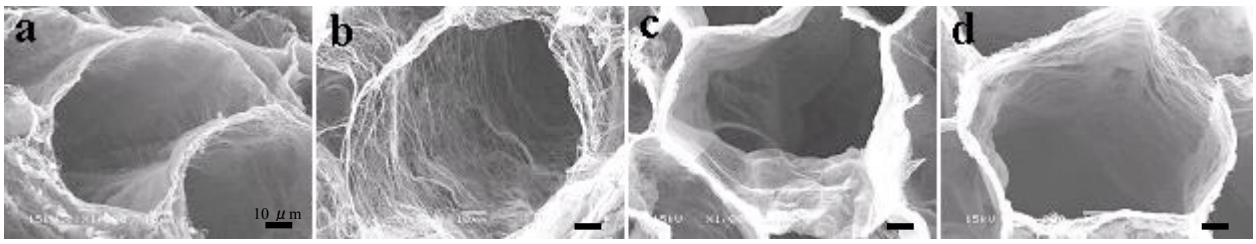

Figure 3. Scanning electron micrographs of the intramuscular connective tissue of enzyme-treated meat. (a) Control. (b) CL-C35-treated meat. (c) CL-AS-treated meat. (d) Papain-treated meat. 1000× resolution.

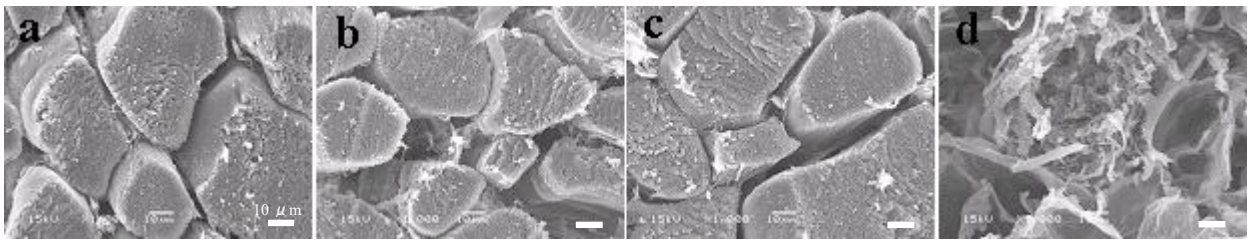

Figure 4. Scanning electron micrographs of the myofibrillar proteins of enzyme-treated meat after heating. (a) Control. (b) CL-C35-treated meat. (c) CL-AS- treated meat. (d) Papain-treated meat. 1000× resolution.

the muscle fibrils of the control, CL-C35-treated and CL-AS-treated meats were arranged in an orderly fashion, whereas the fibrils of papain-treated meat were disrupted and mushy (Fig. 4).

The micrographs showed that CL-AS did not degrade protein at a low temperature; papain degraded both collagen and muscle protein, leading to over-tenderization upon heating; and CL-C35 had high specificity to collagen in the connective tissue framework, likely contributing to desirable meat tenderization.

IV. CONCLUSION

The findings in this study indicate that the new collagenolytic enzyme CL-C35, produced by *Shewanella* sp., holds promise as a meat tenderizer. Unlike the commercially used papain and CL-AS, CL-C35 significantly reduced the shear force value of meat when applied at a low temperature, and showed a marked preference for collagen, which is associated with meat toughness.

However, further work remains to be done before this enzyme can be put to practical use, such as the determination of the sensory acceptability of enzyme-treated meat and the optimal tenderization conditions. Furthermore, a number of points require clarification prior to the large-scale utilization of this enzyme by the meat industry, such as stability during the meat tenderization process and product safety.

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