Effects of Papain and a Microbial Enzyme on Meat Proteins and Beef Tenderness

I.N.A. ASHIE, T.L. SORENSEN, AND P.M. NIELSEN

ABSTRACT: The relative effects of an aspartic proteinase (AP) and papain on meat proteins and beef tenderness were evaluated by measuring release of hydroxyproline in collagen, and breakdown of myofibrillar proteins. Tenderness was objectively measured by Warner-Bratzler shear. AP showed self-limiting hydrolysis of myofibrillar proteins resulting in 25 to 30% improvement in meat tenderness and was not adversely affected by pH, salt, phosphate, and ascorbate concentrations often encountered in meat processing. Like papain, its tenderizing effect was expressed primarily during cooking and caused no significant changes ($p > 0.05$) in tenderness during frozen or refrigerated storage. It was also inactivated at cooking temperatures in excess of 60 °C, therefore eliminating any undesirable side effects that may be associated with residual protease activity.

Keywords: enzyme, tenderness, myofibrillar proteins, collagen, myosin

Introduction

TENDERNESS IS A VERY IMPORTANT QUALITY ATTRIBUTE BY WHICH consumers judge meat quality, and in these times when consumer acceptance for "enhanced" and processed, "meal-ready" meat products is increasing, the tenderness of such products becomes even more significant. Studies have shown that meat tenderness is mainly associated with the structural integrity of myofibrillar and stromal or connective tissue proteins (Marsh and Leet 1966; Smith and Judge 1991; Nishimura and others 1995). The effect of connective tissue on meat tenderness is dependent on the amount, type, and extent of intermolecular cross-linking in collagen, its principal component (Light and others 1985). Since cross-link formation increases as the animal ages, carcasses derived from old animals are generally much tougher than those from young animals having much less collagen cross-linking. Hence, it is common practice to slaughter young animals for meat production. Another common practice in the meat industry is the low-temperature (2 to 4 °C) aging of carcasses to allow proteolysis, resulting in ultrastructural changes in skeletal muscle and, ultimately, improved tenderness (Koohmaraie 1992).

Several other techniques, including mechanical tenderization, elevated-temperature storage, calcium chloride injection, electrical stimulation, muscle stretching, shock-wave pressure, and enzymatic tenderization, have either been investigated or applied to improve meat tenderness (Moeller and others 1976; Savell and others 1981; Koohmaraie 1992; Koohmaraie and others 1988; Cheftel and Culioli 1997). The most widely used enzymes for this application are the plant enzymes papain, bromelain, and ficin (Dransfield and Etherington 1981). Along with microbial proteases derived from *Aspergillus* species, these enzymes have regulatory approval (U.S.D.A) for meat tenderization and have been used in various forms as marinades, injection in brine, pre-slaughter injection into the animal's vascular system, and incorporation into various spices as meat tenderizers (Dransfield and Etherington 1981). However, the market for enzymatic meat tenderizers is still rather small, primarily due to regulatory controls and inability to control the activities of currently used enzymes. Thus far, the only practical means available for controlling the activities of these enzymes is the temperatures attained during cooking, but these have proved inadequate to curtail the activities of widely used enzymes such as papain and bromelain. For example, papain is very heat-stable and, therefore, not readily inactivated, allowing continued product texture deterioration even after cooking (Dransfield and Etherington 1981). Furthermore, these enzymes have very broad specificities and, therefore, indiscriminately break down the major muscle proteins (connective tissue/collagen and myofibrillar proteins), sometimes resulting in overtenderization and a mushy-textured product (Miller and others 1989). Processing facilities in the U.S.A., therefore, currently use either mechanical methods, such as blade tenderization, or natural aging to improve meat tenderness. Except for a few processing facilities, methods such as electrical stimulation or shock-wave pressure are mainly academic and not pervasive in the meat industry.

To ensure controlled breakdown of meat proteins, attempts have also been made to use microbial collagenases with some success (Foegeding and Larick 1986; Miller and others 1989). However, these collagenases have not attracted much interest, due to potential pathogenicity of the enzyme source. Thus, the limited application of enzymes as meat tenderizers may be linked to inherent problems with currently approved enzymes. It is conceivable, however, that this segment could be significantly improved with the development of alternative enzymes with characteristics addressing the problems of the papains, ficins, and bromelains. Cronlund and Woychik (1987) had suggested that an ideal enzyme for meat tenderization may need to have a very narrow specificity, degrading 1 of the major meat proteins but not both, and be able to express its tenderizing effect at the pH encountered during processing and cooking temperatures. This study was, therefore, undertaken to verify the above hypothesis by evaluating the effects of an aspartic protease on meat proteins and the potential impact on beef tenderness.

Materials and Methods

ASPERGILLUS ORYZAE-EXPRESSED ASPARTIC PROTEASE (AP) AND neutrase standard (1.72 Anson units/g) were supplied by Novozymes North America Inc, Franklinton, N.C., U.S.A. Beef top round and briskets were supplied by Southern Foods, Greensboro, N.C., U.S.A., and phosphate was purchased from B.K. Ladenburg Corp, Simi Valley, Calif., U.S.A. Salt and ascorbic acid were purchased from Cargill Foods, Minneapolis, Minn., U.S.A. and Sigma Chemical Co., St. Louis, Mo., U.S.A., respectively.

© 2002 Institute of Food Technologists

Myofibril hydrolysis

Myofibrillar proteins were extracted according to the method of Olson and others (1976) and the protein content determined using a Leco Protein analyzer (Leco Corp., Warrendale, Pa., U.S.A.). Myofibrillar protein breakdown was estimated by the method of Tsai and others (1983). Fifty μL enzyme (1 mg/mL) was added to 1.95 mL of myofibril extract and incubated at 37 °C for 1 h. The reaction was stopped by adding 2 mL of 15% trichloroacetic acid (TCA), and the mixture was allowed to stand at room temperature for 15 min, followed by centrifugation. The absorbance of supernatant was measured spectrophotometrically at 280 nm. The activities of the aspartic protease were compared to that of papain.

Collagen breakdown

Hydrolysis of collagen was measured according to the method of Cronlund and Woychik (1987). We added 0.2 mL enzyme (1 mg/mL) to 20 mg bovine tendon collagen (Sigma Chemical Co.) suspended in 3.8 mL Tris buffer (0.02 M Tris, 0.005 M calcium chloride, pH 7.4); this was incubated at 40 °C for 3 h or 70 °C for 30 min. After this period, the reaction mixtures were spun in a microfuge for 10 min at 14000 rpm. Supernatant (1.5 mL) was mixed with 4.5 mL of 5 N HCl and kept in a drying oven at 110 °C for 16 h for complete hydrolysis of soluble peptides. The hydrolysate obtained was analyzed for hydroxyproline content. Collagenolytic activity of AP was compared to that of papain.

Hydrolysate was diluted 25 times with distilled water and 1 mL chloramine T solution (1.41 g chloramine T dissolved in 100 mL citrate-acetate buffer, pH 6.0) added to 1 mL diluted hydrolysate. The solution was allowed to stand at room temperature for 20 min, 1.0 mL color reagent was added, and it was transferred to a 60 °C water bath where it was further incubated for 15 min. Tubes were allowed to cool, and absorbance was measured at 560 nm.

Sample treatment and evaluation of tenderness

Post-rigor beef top rounds and briskets were randomly injected with varying concentrations (0.002 to 0.05 AU/100 g meat) of AP or papain using a single needle injector (Dayton Electrical Manufacturing Co., Niles, Ill., U.S.A.) until there was a 5% increase in meat weight. The injected meat was tumbled at 5 rpm under vacuum (8 Pa) and 2 to 4 °C to ensure adequate distribution of enzyme within the muscle tissue. The meat was cooked on an electric skillet to internal temperatures of 55 °C, 65 °C, and 75 °C. After cooling to room temperature, tenderness was objectively measured using a Warner-Bratzler shear test on a TAX-T2 texture analyzer (Texture Technologies Corp, Scarsdale, N.Y., U.S.A.). The blade was set to move at a speed of 2.0 mm/s through a fixed distance of 30 mm. The maximum force required to shear the cored sample gives an indication of meat tenderness (inversely related to maximum shear force).

Samples were also evaluated for effect of marinade treatment on tenderness. Beef (top rounds) were sliced 15 mm thick across the length of the fibers, weighed and placed in a vacuum tumbler. 355 g marinade (Lawry's Herb and Garlic with Lemon Juice 30 Minute Marinade) was poured into a graduated cylinder and diluted to 1000 g with distilled water. Percent uptake of marinade by meat was established by adding marinade to a known weight of meat and tumbling for an h at 5 °C. Both meat and marinade were transferred into re-sealable bags and held overnight at 5 °C, after which the meat was re-weighed. Percent uptake was estimated as:

$$\% \text{ uptake} = [(\text{weight of marinated sample} - \text{weight before marination})\text{weight before marination}] \times 100$$

This was found to be 12%; enzyme dose for marinating was calculated based on this and adding enzyme to diluted marinade before transferring to meat in a tumbler. After tumbling for 1 h at 5 °C, meat and marinade were sealed in bags as above and kept under refrigeration prior to cooking on an electric skillet at specified temperatures. Samples were cored after allowing to cool, and tenderness was measured as before.

Residual activity

For residual activity determinations, 0.1 Anson unit (AU) of either AP or papain was added to 100 g ground meat; this was shaped into patties. Controls were also set up by forming patties with equivalent weight of distilled water instead of enzyme. These were cooked to internal temperatures of 55 °C, 65 °C, and 75 °C. The meat was allowed to cool to room temperature, and 10 g was suspended in 40 mL 0.1 M Tris-maleate buffer (pH 5.5). The suspension was homogenized for 1 min using a polytron (Polyscience, Niles, Ill., U.S.A. - model X520) followed by centrifugation at 10000 rpm for 10 min. Residual activity in supernatant was measured by the modified Anson (1939) method.

1.0 mL supernatant was added to 2.0 mL hemoglobin substrate and incubated at room temperature for 10 min. The reaction was stopped by adding 5.0 mL of 0.3 M TCA, and the suspension allowed to stand for 10 min at room temperature. This was filtered and 1.5 mL filtrate added to 2.8 mL of 0.5 M NaOH followed by 1.0 mL Folin–Ciocalteu phenol reagent. After standing for 10 min, absorbance was spectrophotometrically (Hewlett-Packard, Waldbronn, Germany; model HP8453E) measured at 750 nm.

Statistical analysis

Differences between means of treatments were analyzed using JMP statistical software with significance level set at $p \leq 0.05$ (SAS 2000)

Results and Discussion

Meat protein hydrolysis

AP did not hydrolyze collagen and showed very limited hydrolysis of myofibrillar proteins (Figure 1). However, papain showed significant hydrolysis of collagen and about 7-fold activity toward myofibrillar proteins, as compared to AP. Indiscriminate breakdown of meat proteins by papain confirmed reports by other studies (Foegeding and Larrick 1986; Miller and others 1989; Takagi and others 1992). The selective breakdown of meat proteins by AP is also similar to effects of an alkaline elastase isolated from *Bacillus* species (Takagi and others 1992). Unlike AP, however, the alkaline protease had strong activity for selectively cleaving and degrading elastin and collagen, but it had no effect on myofibrillar proteins.

Effect on meat tenderness

Enzymatic hydrolysis of meat proteins is generally known to increase solubilization of free amino groups and hydroxyproline, which may result in loss of muscle integrity and reduced shear force or increased tenderness (Fogle and others 1982). Evaluation of the relative effects of injected AP and papain on tenderness of beef briskets and top rounds showed that maximum shear force was reduced by both enzymes (Figure 2). Papain continued to increase tenderness of both meat portions as its dose was increased. At doses beyond 0.01 AU/100 g meat, papain-treated meat was mushy and could no longer be cored for tenderness evaluation. On the other hand, AP reduced maximum shear force by about 25 to 30% and did not show any further increase in tenderness beyond an enzyme dose of 0.01 AU/100 g meat. Similar effects were observed with marinated beef, but higher doses were required in this case, likely

due to restricted enzyme-substrate contact surface area as compared to the injected meat (Figure 2c). A 10-fold increase in enzyme dose was required to reduce relative shear force by 20% in marinated products. The resistance to further tenderization by AP at higher enzyme concentrations may be attributed to self-limiting hydrolysis as reported by Cronlund and Woychik (1986). These workers had shown that, while papain indiscriminately broke down meat proteins, a microbial protease hydrolyzed myosin to a 130 to 140 kDa band that remained resistant to further hydrolysis.

Processing conditions

A wide range of conditions and ingredients are often used in meat processing, which, therefore, requires that an enzyme meant for meat processing be able to function under these conditions. Furthermore, it also needs to be readily inactivated in order to prevent excessive breakdown of muscle tissue. The effects of various processing conditions on activities of the 2 enzymes were therefore evaluated. It is evident from Figure 3a that AP-induced tenderization was independent of cooking temperatures ranging between 55 to 75 °C, covering "rare", "medium", and "well done" meats. About 20% reduction in shear force was observed at all temperatures compared to controls, thus making it possible to use the enzyme for all the cooking conditions. Residual enzyme activity in meat after cooking was, however, greatly influenced by the cooking temperature. Evaluation of residual activity showed that, while papain retained maximum activity even after cooking at 75 °C, residual AP activity steadily decreased as cooking temperatures increased (Figure 3b). Residual activities were 70%, 28%, and 14% for AP-treated meat heated to core temperatures of 55 °C, 65 °C, and 75 °C, respectively, while there were no significant changes ($p > 0.05$) in residual papain activity at all temperatures. Since high levels of residual activity may continue to affect both flavor and texture characteristics after cooking and serving, it is essential that such activity is as low as possible if not completely inactivated. Furthermore, the self-limiting tenderization by AP coupled with the effect of cooking temperatures on its stability indicates that the tenderizing effect of the enzyme mainly occurs during the initial stages of cooking when the temperature is below that required to inactivate it.

The use of ascorbic acid in meat processing is an age-old practice that has survived the times, and is, therefore, still an integral part of the industry. In an attempt to control over-tenderization of meat by papain, Ockerman and others (1993) demonstrated that ascorbic acid at a concentration of 2.5 mM completely inhibited papain activity, but the level of ascorbic acid used induced off-flavor, rendering the meat unacceptable to sensory panelists. Figure 4a shows that, even at twice this concentration, ascorbic acid had no significant effect ($p > 0.05$) on AP activity. Thus, whereas ascorbic acid may limit the use of papain as a tenderizer, especially in cured meat products, use of AP could potentially broaden the range of such applications.

Reducing the pH below 6.0 had no significant effect on AP activ-

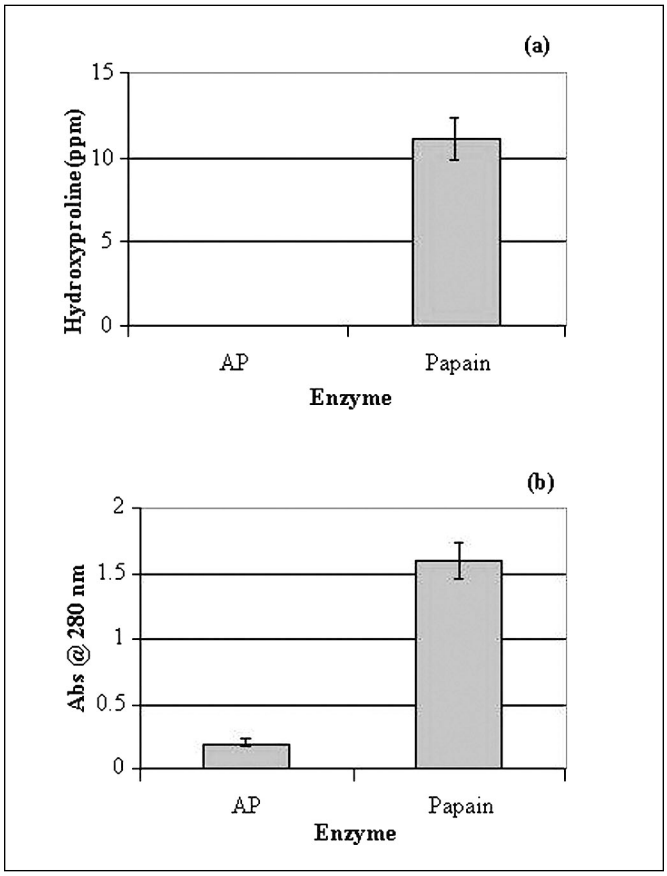

Figure 1–Relative effects of papain and AP on (a) collagen and (b) myofibrillar proteins.

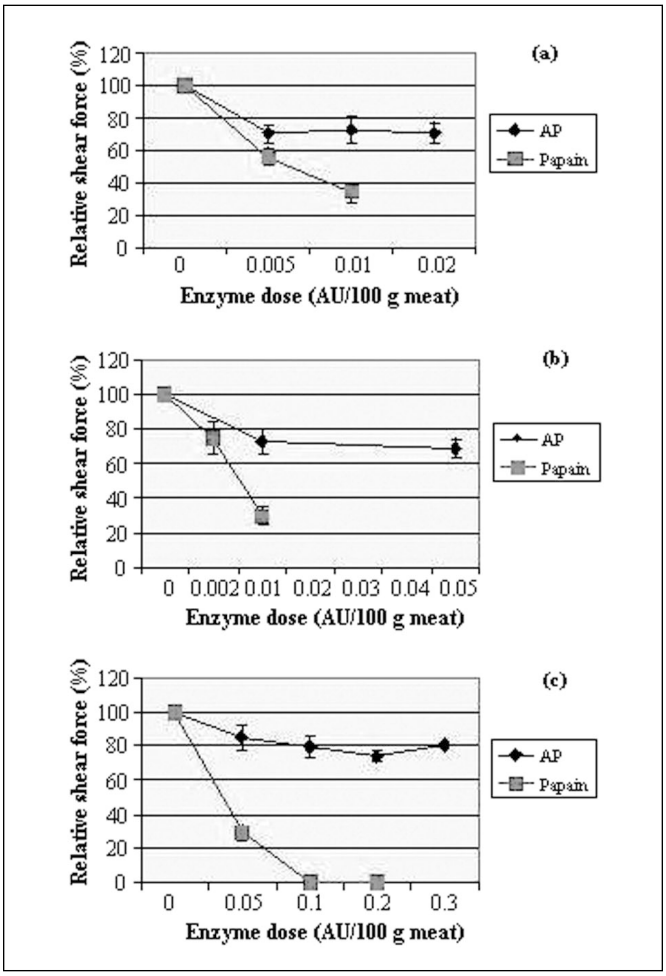

Figure 2–Effect of different doses of papain and aspartic protease on tenderness of beef cuts. (a) and (b) are briskets and top rounds, respectively, treated by injection of enzyme; (c) is marinated top round.

ity (Figure 4b). However, at higher pH (7 and 8), activity was reduced by about 15% and 90%, respectively. While pH of the live animal is well regulated and maintained at about 7.0, postmortem breakdown of the regulatory mechanism results in a gradual decrease in pH to a range of about 5.5 to 6.5, depending on pre-slaughter treatment and physiology of the animal. Hence, in spite of the loss of activity at higher pH, the level of activity at postmortem pH (5.5 to 6.5) is still high enough to bring about the desired improvement in meat tenderness. Figure 4b also shows that in the presence of 0.4% phosphate, which is commonly used in meat products, AP activity was only reduced by about 10 to 15%.

Storage conditions on tenderness

AP also differed from papain in relation to its effects on meat tenderness during storage. Meat treated with either enzyme was stored under refrigeration (5 °C) for 2 wk and the tenderness evaluated after cooking. Papain-treated meat showed gradual reduction in relative shear force during storage, while AP showed no further change in tenderness beyond the first d of storage (Figure 5). Thus, whereas papain-treated meat was being steadily broken down during refrigerated storage, AP showed no significant breakdown ($p > 0.05$) of meat proteins and, therefore, had no effect on meat tenderness. This further supports the suggestion that the tenderizing effect of AP occurs mainly during cooking, a characteristic that could be exploited in the treatment and transportation of various packaged fresh meat products.

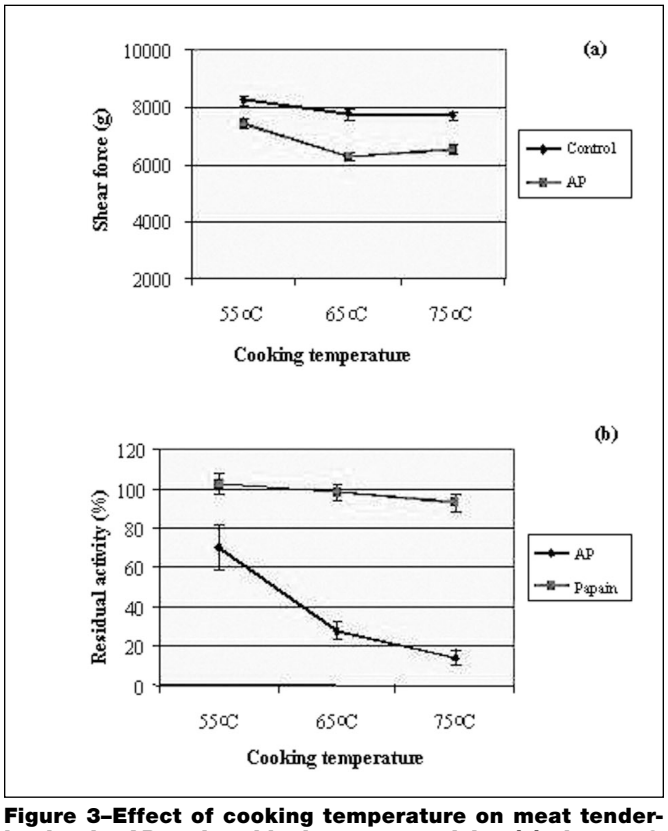

Figure 3–Effect of cooking temperature on meat tenderization by AP and residual enzyme activity. (a) shows effect of AP on tenderness, while (b) shows residual activity after cooking compared to papain under identical conditions.

Conclusions

WHILE THE USE OF ENZYMES AS MEAT TENDERIZERS HAS BEEN LIMITED by the undesirable side effects of approved enzymes like papain and bromelain, the results presented here show that an aspartic protease (AP) expressed in *Aspergillus oryzae* could be used to address the shortcomings of currently approved enzymes. Unlike papain, the new enzyme has very limited specificity on meat pro-

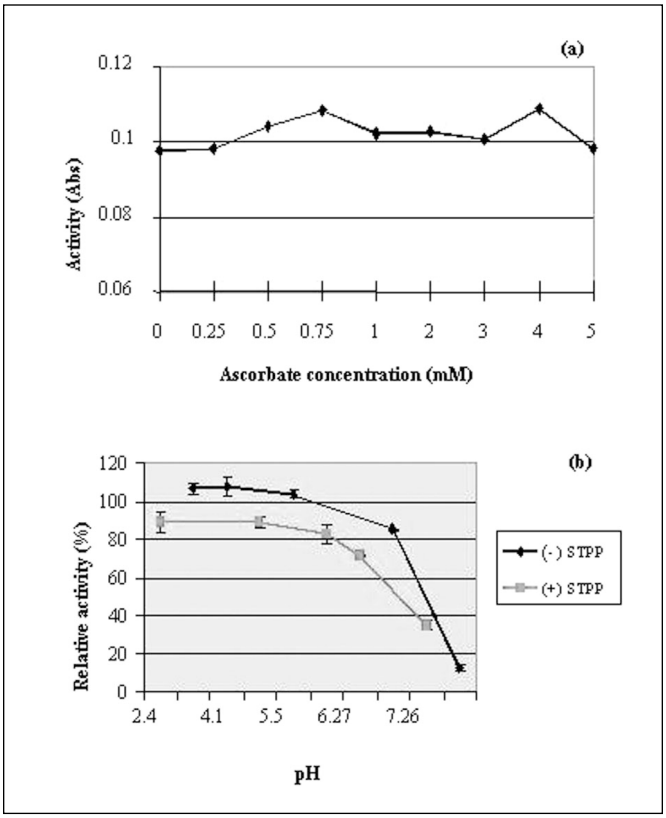

Figure 4–Effect of ascorbic acid, pH and phosphate concentration on AP activity. (a) shows effect of ascorbic acid, while (b) shows phosphate and pH effects. STPP indicates presence (+) or absence (–) of polyphosphates.

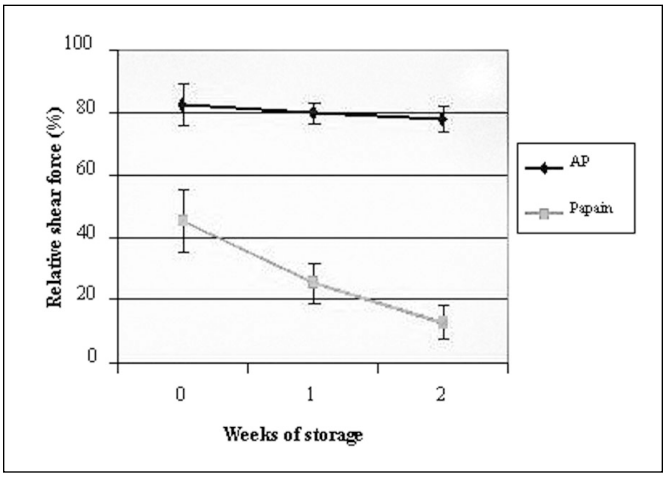

Figure 5–Changes in tenderness of beef treated with papain (0.005 AU/100 g meat) and AP (0.01 AU/100 g meat) during refrigerated storage (5 °C).

teins and acts only on myofibrillar proteins, but not connective tissue; its activity is self-limiting, thereby eliminating the risk of overtenderization. Its effect is primarily manifested during cooking, and could, therefore, be stored without any adverse enzymatic changes in product characteristics; and is also readily inactivated under cooking conditions. With the increased consumer acceptance of "case-ready" meat and other food products, the unique characteristics of AP could be exploited not only for current enzymatic processes, but also provide the innovative meat processor with an invaluable tool for developing new products.

References

Anson ML. 1939. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. J General Physiol 22:79-89.

Cheftel JC, Culioli J. 1997. Effects of high pressure on meat: a review. Meat Sci 5:261-266.

Cronlund AL, Woychik JH. 1987. Solubilization of collagen in restructured beef with collagenase and a-amylase. J Food Sci 52:857-860.

Cronlund AL, Woychik JH. 1986. Effect of microbial rennets on meat protein fractions. J Agric Food Chem 34:502-505.

Dransfield E, Etherington D. 1981. Enzymes in the tenderization of meat. In: Birch GG, Blakebrough N, Parker KJ editors. Enzymes and Food Processing. London: Applied Science Publishers. P 177-194.

Foegeding EA, Larick DK. 1986. Tenderization of beef with bacterial collagenase. Meat Sci 18:201-214.

Fogle DR, Plimpton RF, Ockerman HW, Jarenback L, Persson T. 1982. Tenderization of beef: effect of enzyme, enzyme level, and cooking method. J Food Sci 47:1113-1118.

Koohmaraie M. 1992. The role of Ca^{2+}-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. Biochimie 74:239-245.

Koohmaraie M, Babiker AS, Schroeder AL, Merkel RA, Dutson TR. 1988. Acceleration of postmortem tenderization in ovine carcasses through activation of Ca^{2+}-dependent proteases. J Food Sci 53:1638-1641.

Light N, Champion AE, Voyle C, Bailey AJ. 1985. The role of epimysial, perimysial, and endomysial collagen in determining texture in 6 bovine muscles. Meat Sci 13:137-149.

Marsh BB, Leet NG. 1966. Studies in meat tenderness III. The effects of cold shortening on meat tenderness. J Food Sci 31:450-459.

Miller AJ, Strange ED, Whiting RC. 1989. Improved tenderness of restructured beef steaks by a microbial collagenase derived from Vibrio B-30. J Food Sci 54(4)855-857.

Moeller PW, Fields PA, Dutson TR, Landmann A, Carpenter ZL. 1976. Effect of high temperature conditioning on subcellular distribution and levels of lysosomal enzymes. J Food Sci 41:216-217.

Nishimura T, Hattori A, Takahashi K. 1995. Structural weakening of intramuscular connective tissue during conditioning of beef. Meat Sci 39:127-133.

Ockerman HW, Harnsawas S, Yetim H. 1993. Inhibition of papain in meat by potato protein or ascorbic acid. J Food Sci 58:1265-1268.

Olson GD, Parrish CF, Strowia HM. 1976. Myofibril fragmentation and shear resistance of 3 bovine muscles during postmortem storage. J Food Sci 41:1036-1041.

SAS. 2000. JMP user's guide version 4. SAS Institute Inc., Cary, N.C., U.S.A.

Savell JW, McKeith FK, Smith GC. 1981. Reducing postmortem aging time of beef with electrical stimulation. J Food Sci 46:1777-1781.

Smith SH, Judge MD. 1991. Relationship between pyridoline concentration and thermal stability of bovine intramuscular collagen. J Anim Sci 69:1989-1993.

Takagi H, Kondou M, Hisatsuka T, Nakamori S, Tsai YC, Yamasaki M. 1992. Effects of an alkaline elastase from an alkalophilic Bacillus strain on the tenderization of beef meat. J Agric Food Chem 40:2364-2368.

Tsai YC, Yamasaki M, Yamamoto-Suzuki Y, Tamura G. 1983. A new alkaline elastase of an alkalophilic *Bacillus*. Biochem Int 7:577-583.

MS 20010595 Submitted 10/22/01, Accepted 12/3/01, Received 12/3/01

The authors are with Applied Discovery (Meat & Dairy Applications), Novozymes North America, Franklinton, NC 27525-0576. Direct inquiries to author Ashie (E-mail: Isaa@novozymes.com)