Research Article

Received: 22 January 2009 Revised: 26 March 2009 Accepted: 14 April 2009 Published online in Wiley Interscience: 22 May 2009

(www.interscience.wiley.com) DOI 10.1002/jsfa.3633

Injection of marinade with actinidin increases tenderness of porcine *M. biceps femoris* and affects myofibrils and connective tissue

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Abstract

BACKGROUND: Marination of beef muscles with brine solutions containing proteolytic enzymes from fruit extracts has been shown to tenderize meat. However, the effect of marination with actinidin on tenderness of pork muscles has not been investigated. Tenderness and eating quality of porcine *M. biceps femoris* was investigated by Warner–Bratzler (WB) shear test and sensory evaluation after injection of brine containing up to 11 g L^{-1} actinidin-containing kiwi fruit powder and 2, 5 or 9 days of storage.

RESULTS: Actinidin decreased WB shear force, increased tenderness and did not affect flavour and juiciness. Injection of 2.8 g L^{-1} actinidin powder and storage for 2 days resulted in WB shear force values similar to control samples stored for 5 or 9 days. In samples injected with 10 g L^{-1} actinidin powder, degradation of desmin and percentage of heat-soluble collagen ($P < 0.05$) increased compared to control samples. Myofibrillar particle size tended to decrease ($P < 0.1$) with increasing actinidin concentration. No major changes were observed by proteome analysis. Atomic force microscopy showed actinidin-induced damage of endomysium surrounding isolated single muscle fibres.

CONCLUSION: Our results indicate that actinidin tenderizes pork *M. biceps femoris* by affecting both the myofibrils and connective tissue.

Keywords: actinidin; tenderness; pork; atomic force microscope; collagen solubility; desmin

INTRODUCTION

Tenderness is a very important eating quality attribute in beef, lamb and pork. Large muscle-to-muscle variations in tenderness exist, giving rise to economic diversities between different muscles within the same animal. Marination of meat with brine solutions containing proteolytic enzymes from fruit extracts, such as ficin from fig,[1] papain from papaya fruit[1–4] and bromelain from pineapple[2] have been used to tenderize meat.

Actinidin is a cysteine protease present in kiwi fruit and it belongs to the same class of enzymes as ficin, papain and bromelain.[5,6] The tenderizing effect of actinidin and papain using bovine *semitendinosus* muscle was compared.[7] The authors found that a higher activity of actinidin was needed in order to produce tenderness equivalent to that observed using papain. Furthermore, actinidin did not over-tenderize the meat, myosin heavy chain was degraded and changes in the band intensity of other myofibrillar proteins was reported. Actinidin did not produce off-flavours or odours in the meat and no surface 'mushiness' was found. Collagen is affected by the enzyme as incubation of bovine Achilles tendon with purified actinidin either at neutral or acidic pH increased the solubility of collagen.[8] Also, cross-linked collagen β- and γ-chains isolated from beef shank were converted to α-chains after incubation in kiwifruit juice at acidic pH.[9] The authors suggested that kiwifruit juice might degrade the globular domains of collagen which are involved in intra- and intermolecular cross-linking. Furthermore, incubation in kiwifruit juice resulted in reduced shear force of connective tissue. Recently, actinidin was found to hydrolyse collagen type I and II under neutral and alkaline conditions.[10] Vascular infusion of kiwifruit juice containing actinidin into lamb *longissimus dorsi* muscle reduced shear force and lipid oxidation.[11] Although previous studies show that actinidin tenderizes beef and lamb muscles, a clear understanding of the structural changes involved in actinidin-induced tenderisation is still lacking and no studies showing the tenderizing effect of actinidin on porcine muscles with high connective tissue content has been reported.

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The objective of the present study was to investigate the effect of injection with actinidin from kiwifruit on the tenderness and eating quality of porcine *M. biceps femoris*. Actinidin-induced changes in the myofibrils and connective tissue were evaluated by analysing Warner–Bratzler (WB) shear pattern, sensory attributes, myofibrillar fragmentation, proteome analysis, degradation of desmin, ultrastructure of single muscle fibres and percentage of heat-soluble collagen.

EXPERIMENTAL

Animals and muscle samples

Experiment 1

Biceps femoris muscles were excised 24 h post mortem from both sides of 30 female pigs. The pigs were slaughtered according to standard slaughtering procedures at a commercial slaughterhouse. Carcasses were selected on the basis of slaughter weight (74–78 kg), lean meat percentage (57–60%) and pH 24 h post mortem (5.55–5.65). Muscles were vacuum-packed and stored at 2 °C until 48 h post mortem, then weighed and injected using a multi-needle injector (Fomaco model: FGM 48 SC, Food Machinery Co., Rochester, UK) with settings of 0.9 bar, 66 strokes min^{-1}, 3 bar up-pressure). Five brines were prepared by mixing a neutral marinade composed of a mixture of maltodextrin (81%) and starch (19%) (India-dan, Randers, Denmark) with actinidin-containing kiwifruit powder (OT-1005X, Ingredient Resources, Warriewood, Australia). The composition of the five marinades were 93 g L^{-1} neutral marinade plus 0, 2.8, 5.5, 8.3 and 11 g L^{-1} actinidin powder. pH of the brine solutions were recorded and purity of the kiwifruit extract was evaluated by running a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel (Fig. 1). Both left and right side muscles from six animals were injected with each of the five brine solutions to an average weight gain of 16%. After injection the weight was recorded to calculate the weight gain, and muscles were vacuum-packed and then stored for 48 h at 2 °C. At day 4 post mortem left side muscles were frozen and stored at −20 °C until sensory assessment. Right side muscles were weighted to calculate the injection loss, pH was recorded and then the muscles were divided into three pieces of identical size: one piece was frozen immediately (corresponding to day 4 post mortem) and the two other pieces were vacuum-packed and then stored for 3 or 7 days at 2 °C (corresponding to day 7 and 11 post mortem, respectively) before freezing at −20 °C. These samples were used for WB shear force measurements.

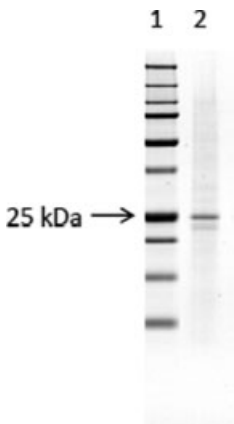

Figure 1. Characterization of actinidin by SDS-PAGE. 10 mg actinidin-containing kiwi fruit powder was dissolved in 180 µL of sample buffer and heated at 70 °C for 10 min. After centrifugation reducing agent was added to an aliquot of the supernatant and 10 µg of protein was loaded onto the gel (lane 2). One protein band at 25 kDa is clearly seen and a less intense band at 23 kDa is also visible. Only trace amounts of other proteins can be observed. The first lane shows molecular weight standards (Precision Plus Protein Standards, Bio-Rad) of 250, 150, 100, 75, 50, 37, 25 (arrow), 20, 15 and 10 kDa.

Experiment 2

Biceps femoris muscles were excised from five female pigs following the same procedures as described above. At 48 h post mortem each muscle was divided into smaller pieces of approximately identical size. Each piece was weighed and then injected with one of three brine solutions containing 93 g L^{-1} neutral marinade plus 0, 4 and 10 g L^{-1} actinidin-containing kiwifruit powder using a multi-channel pipette (Eppendorf, AH diagnostics, Aarhus, Denmark) consisting of eight disposable needles (1.5 × 50 mm) to obtain 10% weight gain. The samples were injected evenly along and across the muscle pieces to avoid uneven distribution of the brine. Furthermore, effect of sample position in the muscle was accounted for by injecting each brine solution at the five sample positions. In total, each combination of brine and position was repeated 10 times. After injection the weight of the muscle piece was recorded to calculate the weight gain. Each piece was vacuum-packed and stored for 48 h at 2 °C, and then divided into smaller samples for determination of myofibrillar fragmentation (10 g), degradation of desmin (10 g), two-dimensional electrophoresis (10 g), atomic force microscopy (10 g) and total collagen content as well as percentage heat-soluble collagen (80 g). Samples for myofibrillar fragmentation, desmin degradation, two-dimensional electrophoresis and atomic force microscopy were frozen and stored at −80 °C until use. Samples for collagen characteristics were frozen and stored at −20 °C until use.

Warner–Bratzler shear force

Meat toughness was determined using the WB shear device. Prior to analysis, right side muscles (experiment 1) were thawed 24 h at 2 °C, the weight recorded to calculate thaw loss and then samples were heated at 75 °C for 1 h in a circulating water bath. Cooking was arrested by placing the samples in ice-cold water for 1 h. The weight of the samples was recorded again to calculate the cooking loss. Four rectangular shaped blocks (1 × 1 × 5 cm) were cut and each block was sheared three times perpendicular to the muscle fibre direction with a triangular-shaped shear blade on an Instron Universal testing machine (High Wycombe, UK). The average of 12 shear values represented the WB shear force value of one animal. In total, six animals per brine solution were measured. The crosshead speed was 50 mm min^{-1}. The maximum load required to shear through the sample (WB peak force) was determined.

Sensory assessment

Biceps femoris muscles from the left side (experiment 1) were thawed at 3–4 °C for approx. 22 h. After thawing, the muscles were equilibrated at room temperature (approx. 20 °C) to an internal temperature of a maximum of 15 °C. The muscles were placed in roasting bags and cooked in a convection oven at 100 °C to a core temperature of 75 °C. The roasts were cut into 10 mm thick slices, and half a slice was served on a pre-heated plate to each assessor. Sensory assessment of texture, taste and flavour was performed in two sessions on different days. Samples were evaluated by nine trained assessors using a 15-point non-structured line anchored at the extremes (0 = slight and 15 = intense). The attributes were:

meat flavour (intensity of pork flavour), warmed-over flavour (intensity of perception of reheated pork), metal flavour (intensity of perception of metallic taste, like a coin), piggy flavour (intensity of warm pork fat), hardness (hardness at one bite with the back tooth), juiciness (amount of meat juice after five to six times of chewing), crumbliness (amount of meat dust during chewing), threadiness (amount of shred of meat), chewing time (the time it takes to chew a sample before it is ready to be swallowed) and tenderness (how easy it is to divide the meat during chewing).

Myofibril fragmentation

This procedure followed the methodology described earlier.[12] Briefly, muscle tissue (2.5 g) from experiment 2 was chopped into small pieces and immediately transferred into 30 mL cold homogenization buffer (100 mmol L^{-1} KCl, 20 mmol L^{-1} potassium phosphate (pH 7.0), 1 mmol L^{-1} EGTA, 1 mmol L^{-1} $MgCl_2$). Samples were homogenized at 20 500 rpm for 30 s using an Ultra-Turrax T25 equipped with a S25N-18 G dispersing element (Ika Labortechnik, Staufen, Germany) and then kept on ice until measurement. Each homogenization was performed in duplicate. Sizes of myofibrils were measured using a Malvern Mastersizer Micro Plus (Malvern Instruments Ltd, Malvern, UK).

SDS-PAGE and immunoblotting of myofibrils

Muscle samples (2 g) from experiment 2 were homogenized in 20 mL cold extraction buffer (50 mmol L^{-1} MES (pH 5.6), 5 mmol L^{-1} EDTA) for 45 s at 13 500 rpm using an Ultra-turrax T25 (Ika Labortechnik). The homogenate was centrifuged at 4 °C for 5 min at 4000 × *g*. The supernatant was removed and 20 mL of cold extraction buffer was added to the pellet and mixed. The samples were centrifuged again and the supernatant was then removed. Cold extraction buffer (10 mL) was added again and the sample mixed thoroughly. SDS at a final concentration of 1% was added and samples were then stored at −20 °C. Protein concentration was determined using the BCA protein determination kit (Pierce, Rockford, IL, USA). Muscle samples were prepared for SDS-PAGE by addition of NuPage LDS sample buffer (Invitrogen, Carlsbad, CA, USA). NuPage reducing agent (Invitrogen) was added to the samples prior to SDS-PAGE and immunoblotting. The final concentration of protein in the samples was 1.5 mg mL^{-1}.

Degradation of desmin was detected by performing SDS-PAGE and immunoblotting as described earlier,[13] but with slight modifications. The monoclonal antibody, mouse anti-desmin (DE-R-11, 1 : 5000, Dako, Glostrup, Denmark) was used as the primary antibody. The secondary antibody was anti-mouse IgG horseradish peroxidase linked whole antibody (1 : 5000, Amersham Biosciences, Little Chalfont, UK). Antibody binding was visualized by ECL plus kit (Amersham Biosciences). Densitometric scans (Umax powerlook 1120, Milton Keynes, UK) of immunoblots were performed using a Phoretix software program (Phoretix, Newcastle Upon Tyne, UK). The mean peak areas of native desmin were determined at each actinidin concentration. The mean peak area of desmin in samples marinated without actinidin (control) was set to 100% and the mean peak area of native desmin in samples marinated with actinidin was expressed as percentage desmin remaining compared to the control samples.

Proteome analysis

Control samples without actinidin and samples injected with 10 g L^{-1} actinidin-containing kiwifruit powder (experiment 2) were analysed using two-dimensional electrophoresis (2DE). One gram of muscle tissue was homogenized in 10 mL ice-cold buffer (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol, 0.2% Biolyte 3–10 (Bio-Rad, Hercules, CA, USA) on ice with an Ultra Turrax T25 (Ika Labortechnik) 2 × 30 s at 9500 rpm followed by 2 × 30 s at 13 500 rpm. The samples were centrifuged at 4 °C for 60 min at 20 000 × *g*, and the supernatant was removed for 2DE and stored at −80 °C until use. 11 cm, pH 4–7 IPG strips (11cm Bio-Rad) were used for the first dimension and 4 µL of sample was loaded during the rehydration step. In the second dimension 10–20% Tris–HCl gel (Criterion, Bio-Rad) was used. The gels were stained with SYPRO Ruby (Molecular Probes, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual and the fluorescence stained proteins were detected with a CCD camera (Raytest, Camilla II, Strubenhardt, Germany). ImageMaster 2D Platinum software v5 (Amersham Bioscience, Uppsala, Sweden) was used for image analysis. The spot intensity was measured as the relative volume within a gel representing normalized values that remain relatively independent of irrelevant variation between gels.

Atomic force microscopy of single muscle fibres

Single muscle fibres were isolated from thin strips of frozen muscle from control samples and samples injected with 10 g L^{-1} actinidin-containing kiwifruit powder as described in a previous study.[14] Immediately after isolation, the single fibre was incubated 5 min in a fixative containing 4% formaldehyde and 1% methanol in PBS (Dulbecco's phosphate-buffered saline) (Sigma Aldrich Co. Ltd, Gillingham, UK). After the fixation the fibre was placed on a piece of polished silicon wafer and fixed with a small strip of tape, washed carefully with MilliQ water and dried with argon. Atomic force microscope (AFM) investigations of muscle fibres were performed with a PSIA XE 150 microscope (Park Systems, Suwon, Korea) in non-contact mode using a BS-Tap300AL cantilever (Budget Sensors, Sofia Bulgaria) (resonant frequency 300 kHz, force constant 40 N m^{-1}) under ambient conditions. For each treatment a minimum of six muscle fibres were investigated.

Total collagen content and percentage heat-soluble collagen

Heat-insoluble and heat-soluble collagen was determined as described.[15] Briefly, the concentration of hydroxyproline was determined as described earlier.[16] A factor of 7.14 was used to convert hydroxyproline to collagen. The amount of soluble collagen was calculated from the hydroxyproline concentration in the supernatant and expressed as a percentage of the total collagen content. The total collagen content was calculated from the sum of the hydroxyproline concentration in the pellet (insoluble collagen) and in the supernatant (soluble collagen) and expressed in milligrams per gram wet tissue.[16]

Statistical analysis

Statistical evaluation of WB shear force data and sensory data was performed using the Procedure Mixed in SAS (Ver. 9.1, SAS Institute Inc., Cary, NC, USA). Degrees of freedom were estimated with the Satterthwaite method, as the material was unbalanced. The main effect of actinidin concentration and storage time (WB shear force only) was determined. The statistical model included the fixed effect of actinidin concentration, storage time and the interaction between fixed effects. Weight gain after injection was included as a covariate. Animal within actinidin concentration and assessors (sensory only) were considered as random effects. Significant differences between least-square

means of the interaction between actinidin concentration and storage time were evaluated using the option Pdiff. For myofibrillar fragmentation, disappearance of native desmin, total and heat-soluble collagen the statistical model included the fixed effect of actinidin. Animal and side of carcass were considered random effects. The principal component analysis (PCA) was generated using the program SenPaq 4.1, calculating a correlation matrix PCA, equivalent to standardizing variables to zero mean and unit variance. Evaluation of the proteome analysis data were performed by a Student *t*-test (ImageMaster 2D Platinum software v5).

Values were considered significantly different if $P < 0.05$.

RESULTS

Changes in pH and weight gain after injection, marination loss, thaw loss and cooking loss in *biceps femoris* muscle

In experiment 1 the weight gain after injection of brine solution was 16% ± 2% for all brine solutions. Approximately 10% ± 1% of muscle juice was lost (marination loss) after storage of injected samples for 2 days at 2 °C. Freezing and subsequent thawing of the samples led to 8% ± 1% weight losses (thaw loss). Finally, heating the muscle samples for WB shear force measurements resulted in around 30% ± 1% cooking loss.

pH decreased from 7.6 in the control marinade to 5.3 in the brine solution with 10 g L^{-1} actinidin powder. However, pH in the meat was not affected by the injection of brine solution since pH prior to injection of marinade was 5.54 ± 0.02 and after injection was 5.56 ± 0.04 ($n = 5$).

Actinidin-induced changes in toughness and flavour

Significant main effects of actinidin ($P < 0.001$) and storage time ($P < 0.001$) were found for WB peak force. No significant interaction between brine solution and storage time was found.

Table 1 shows changes in WB peak force (N) with increasing actinidin concentration and subsequent storage. At each storage time, WB peak force was significantly lower following actinidin injection. At 2 days of storage, samples injected with 11 g L^{-1} actinidin powder had significantly lower WB peak force than samples injected with 0 and 2.8 g L^{-1} but did not differ from samples injected with 5.5 and 8.3 g L^{-1}. At 9 days of storage, samples injected with 11 g L^{-1} actinidin powder had significantly lower WB peak force than samples injected with 0, 2.8 and 5.5 g L^{-1}. Storage to day 9 increased tenderness of control and actinidin-marinated samples.

Table 1. WB peak force (N) of porcine *biceps femoris* injected 48 h post mortem with brine solution containing a neutral marinade (maltodextrin and starch) in combination with different concentrations of actinidin. After injection, samples were stored at 2 °C for further 2, 5 and 9 days. Values represent least-square means and standard error of the mean (SEM) ($n = 6$)

	Storage after injection (days)			
Actinidin (g L^{-1})	2	5	9	SEM
0	38.7a	35.6ab	32.7bc	2.5
2.8	29.1bcd	24.7def	24.0ef	2.3
5.5	27.0cde	24.5defg	22.8defg	2.5
8.3	22.5defh	20.2efgh	17.1gi	2.4
11	21.4efg	20.7fgh	16.2hi	2.4

Mean values without a common letter (a–i) differ ($P < 0.05$).

As shown by principal component analysis (Fig. 2), PC 1 explains 95.5% of the variation and is distended by textural attributes, with tenderness and crumbliness to the left and hardness, threadiness and chewing time to the right. Only 2.3% of the variation is explained by PC2, which is distended by some minor variation in taste and flavour. The samples were divided into three groups (on PC1) depending on the actinidin concentration. Placed furthest to the right, control samples constitute the group characterized by

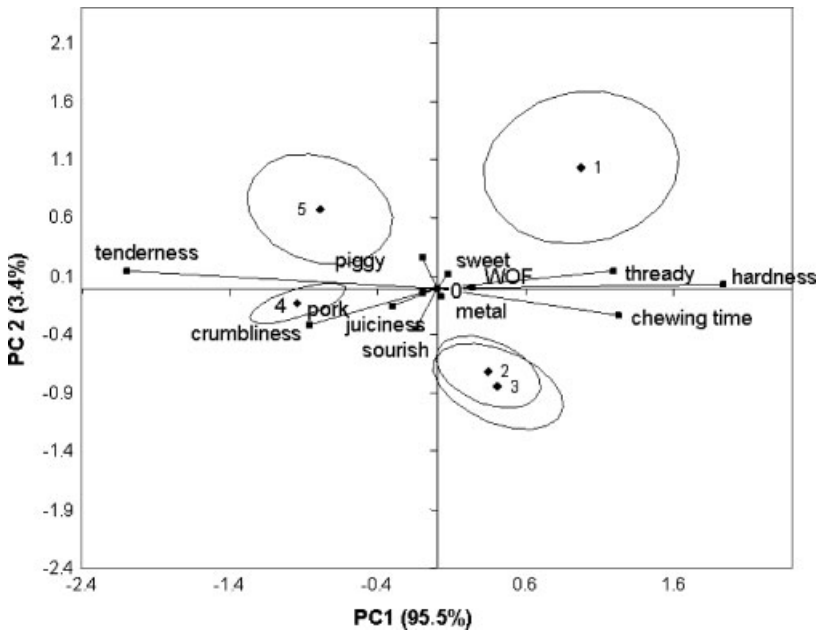

Figure 2. PCA plot showing the actinidin concentration in the brine solution and sensory quality attributes. Levels: 1 = 0 (control), 2 = 2.8 g L^{-1}, 3 = 5.5 g L^{-1}, 4 = 8.3 g L^{-1}, 5 = 11 g L^{-1} actinidin-containing kiwifruit powder; sensory quality attributes: sourish, sweet taste, piggy flavour, meat flavour, warmed-over flavour, metal flavour, hardness, juiciness, crumbliness, threadiness, chewing time and tenderness.

Table 2. Eating quality attributes of sensory assessed porcine *biceps femoris* injected with brine solutions containing varying concentrations of actinidin and subsequently stored for 2 days at 2 °C. Samples were evaluated by nine trained assessors using a 15-point non-structured line anchored at the extremes (0 = slight and 15 = intense)

	Actinidin (g L^{-1})					
Attributes	0	2.8	5.5	8.3	11	*P*
Tenderness	6.2a	7.6a	7.6a	10.8b	10.8b	<0.001
Hardness	7.5b	6.0b	6.1b	3.1a	3.3a	<0.001
Chewing time	9.2a	8.7a	8.9a	6.6b	6.8b	0.002
Threadiness	4.7b	3.4b	3.1ab	1.8a	1.7a	0.003
Crumbliness	6.0a	7.3ab	7.2ab	8.5b	7.8b	0.005
Juiciness	2.8	2.9	2.9	3.2	2.9	0.82
Pork flavour	6.2	6.7	6.1	7.2	6.3	0.07
Warmed-over flavour	3.0	2.8	2.9	2.3	2.6	0.45
Metal flavour	1.5	1.7	1.8	1.6	1.6	0.90
Piggy flavour	2.5	1.7	1.8	2.5	2.2	0.48
Sweet taste	2.5	2.3	2.3	2.1	2.5	0.44
Sourish taste	4.6	5.5	5.9	5.5	5.4	0.24

Values without a common letter (a, b) differ ($P < 0.05$).

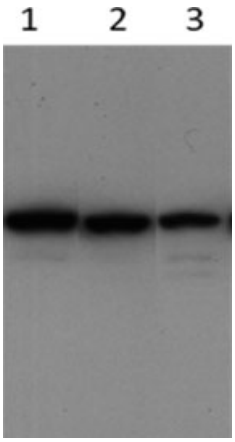

Figure 3. Western immunoblot of desmin in injected porcine *biceps femoris*. Myofibrils were isolated from muscle samples injected with 0, 4.0 or 10 g L^{-1} actinidin-containing kiwifruit powder. 15 µg of protein was loaded onto the gel. Immunoblotting using mouse anti-desmin as the primary antibody and anti-mouse IgG horseradish peroxidise linked whole antibody as the secondary antibody was performed. Native desmin and its degradation products were visualized by ECL plus kit. The brine consisted of maltodextrin and starch containing 0 (lane 1), 4.0 (lane 2) or 10 g L^{-1} (lane 3) actinidin-containing kiwifruit powder.

Table 3. Effect of injecting porcine *biceps femoris* muscle with a brine solution containing varying amounts of actinidin on collagen characteristics, percentage native desmin[a] and average particle size of myofibrils expressed as *D*(*v*, 0.1).[b] Values are expressed as least-square means ± SE ($n = 10$ for control samples and $n = 20$ for actinidin-injected samples)

Actinidin (g L^{-1})	0	4.0	10
Total collagen content (mg g^{-1} wet tissue)	3.9 ± 0.2	3.8 ± 0.1	3.8 ± 0.1
Soluble collagen (%)	31.8 ± 1.4a	33.9 ± 1.2ab	34.3 ± 1.2b
Native desmin (%)	100 ± 11a	91 ± 10ab	76 ± 12b
D(*v*, 0.1) (µm)	16.5 ± 2.2	16.3 ± 1.9	13.0 ± 1.9

[a] Percentage remaining native desmin is expressed as percentage of the desmin content in samples injected without actinidin.
[b] *D*(*v*, 0.1) is the size of the particles for which 10% of the sample is below this size.
Within traits, means without a common letter (a, b) differs ($P < 0.05$).

hard and thready texture with the longest chewing time. Placed further to the left, samples with 2.8 and 5.5 g L^{-1} actinidin powder constitute another group characterized by a more tender and crumbly texture compared with controls. The third group consists of samples 4 and 5, which were injected with 8.3 and 11 g L^{-1} actinidin powder, respectively. These samples are characterized by a significantly more tender and crumbly texture. Table 2 indicates that tenderness, hardness, chewing time, threadiness and crumbliness are affected ($P < 0.01$) by actinidin. However, only pronounced effects were observed with the two highest concentrations of actinidin. Taste, flavour and juiciness were not significantly affected by actinidin injection.

Actinidin-induced changes in the myofibrillar and connective tissue component

Table 3 shows the least-square mean values of total collagen content, heat-soluble collagen, particle size of myofibrils (*D*(*v*, 0.1)) and percentage of native desmin remaining after injection and subsequent storage at 2 °C for 2 days. Comparing samples injected with 10 g L^{-1} actinidin powder to control samples without actinidin showed decreased band intensity of native desmin (Fig. 3) and increased percentage of heat-soluble collagen ($P < 0.05$). The particle size of myofibrils tended to decrease ($P < 0.1$) with increasing concentration of actinidin. Only a few protein spots were found to be significantly different between samples injected with actinidin compared to control samples in the proteome analyses (Fig. 4), suggesting that the muscle proteins were affected to some degree but without being extensively degraded. Around 400 spots were annotated on each 2DE gel.

Figure 5 shows high-resolution AFM images (scale bar 2 µm) of representative single fibres isolated from control (Fig. 5(A)) and actinidin-injected muscle (Fig. 5(B)). In controls, the collagen fibres of the endomysium appeared intact. In contrast, fibres isolated from actinidin-injected samples showed a rough, damaged surface.

DISCUSSION AND CONCLUSION

A limited number of studies have reported a tenderizing effect of kiwifruit protease (actinidin) in bovine[7,17] and lamb[11] muscles. The changes induced by injection of actinidin into porcine *M. biceps femoris* followed by storage for 2 days at refrigerated temperatures clearly demonstrates that this treatment efficiently reduce toughness in a dose-dependent manner. In agreement, the sensory analysis revealed that texture attributes were improved by actinidin injection whereas juiciness, flavour and taste were not affected. This shows that actinidin can be applied as a meat tenderizer without affecting other sensory attributes. In compliance, it was reported that marination with actinidin did not produce off flavours or odours in beef *semitendinosus*.[7]

Considerable amounts of muscle juice were lost from injected samples within the first 2 days of storage. Nevertheless, actinidin appeared to be active and present within the meat even after prolonged storage up to 9 days after injection. Visual inspection

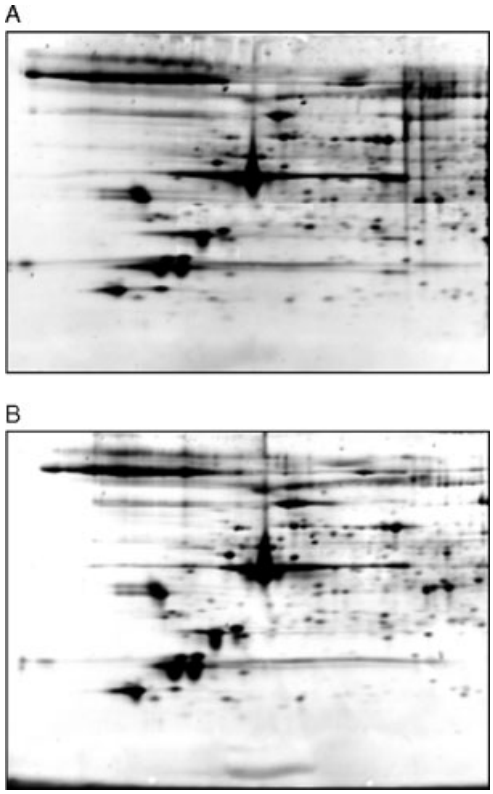

Figure 4. Two-dimensional gel electrophoresis of injected porcine *biceps femoris*. Muscle homogenates from samples injected with either 0 or 10 g L^{-1} actinidin-containing kiwifruit powder were prepared and the supernatant was removed for 2DE. 11 cm, pH 4–7 IPG strips were used for the first dimension and 4 μL of sample was loaded during the rehydration step. In the second dimension 10–20% Tris-HCl gel was used. Protein spots were visualized using SYPRO Ruby followed by detection with CCD camera. The brine consisted of maltodextrin and starch containing 0 (A) or 10 g L^{-1} (B) actinidin-containing kiwifruit powder.

suggested that storage for 9 days did not result in a mushy surface texture as reported for papain.[7] In accordance, these authors also did not observe surface 'mushiness' in actinidin marinated bovine *semitendinosus* muscle. Although tenderization was proceeding up to day 9, the present study shows that actinidin accelerates tenderization. Thus WB peak force values in samples injected with low levels of actinidin and then stored for 2 days were similar to control samples stored for 9 days. This study therefore suggests that injection with actinidin can produce tender pork even in tougher porcine muscles.

Lysosomal enzyme activation by acid marination of beef was shown to increase meat tenderness through an effect on both myofibrillar proteins and on the connective tissue.[18,19] To evaluate the mechanism of actinidin-induced tenderization in porcine *biceps femoris*, specific analysis of changes in the myofibrils and connective tissue was performed. The data presented here suggest that the mechanism of actinidin-induced tenderization is a combination of weakening of the connective tissue and the myofibrils.

The first evidence for actinidin-induced weakening of the myofibrillar structure was supplied by WB shear force. In meat samples cooked at 80 °C, the force at initial yield can be used to evaluate changes in the myofibrillar component.[20] In compliance, the breaking strength of single muscle fibres isolated from porcine *longissimus dorsi* increased at temperatures above 65 °C.[21] Later it was concluded that changes in WB peak force at temperatures above 60 °C was caused mainly by the increased strength of the myofibrillar component.[22] In the present study, muscle samples were cooked at an internal temperature of 75 °C. Thus, it is speculated that the changes in peak force reflect changes in the myofibrillar component. In control samples WB peak force was found to decrease with storage time. It is well established that the myofibrillar component undergoes proteolysis during post-mortem storage which results in decreased toughness,[23,24] again indicating that WB peak force does reflect changes in the myofibrils. The observed actinidin-induced decrease in WB peak force is consistent with the decrease in sensory scores for hardness (hardness at first bite).

Measurement of myofibril fragmentation has been widely used to determine post-mortem proteolysis in meat,[25–27] and myofibrillar fragmentation is a useful indicator of the extent of myofibrillar protein degradation.[25] Marination of bovine *longissimus dorsi* with *Sarcodon aspratus* proteases was observed to increase myofibrillar fragmentation.[28] Multi-angle light scattering has been used to determine differences in particle size distributions

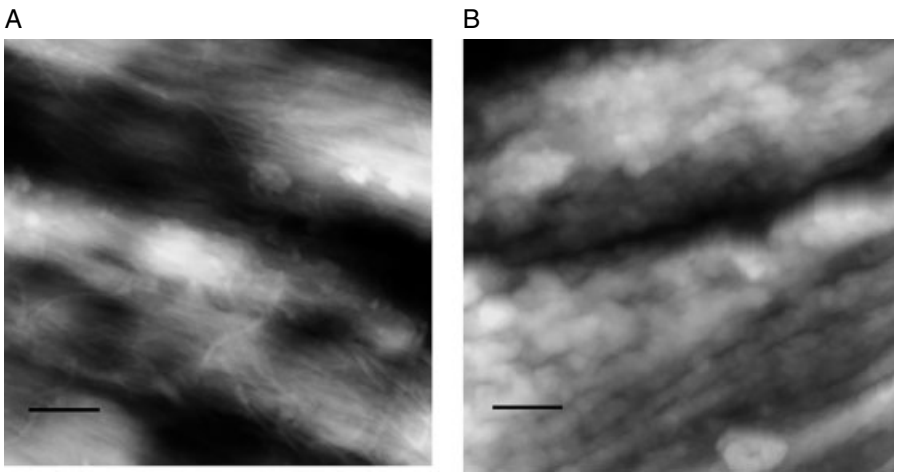

Figure 5. A representative high-resolution atomic force microscope image of a single muscle fibre isolated from porcine *biceps femoris* injected with a brine solution without actinidin (control) (A) and actinidin-containing kiwifruit powder (10 g L^{-1}) (B). In image A collagen fibres are clearly seen, in contrast to image B, which shows a rough surface of the muscle fibres with damaged endomysium. Scale bar = 2 μm.

between unaged and aged samples of pork.[12] In the present study, only a tendency towards actinidin-induced fragmentation of myofibrils was seen, suggesting that actinidin injection did not result in pronounced fragmentation of myofibrils. In accordance, data from the proteome analysis revealed no extensive proteolysis.

Actinidin-induced degradation of myosin but also degradation of other myofibrillar proteins has previously been reported.[7] However, these authors stated that actinidin did not hydrolyse the proteins to the same extent as papain. The observation that actinidin did not degrade the myofibrillar proteins to a large extent is in accordance with our results and might explain why actinidin-injected samples did not develop surface 'mushiness'. In the present study, degradation of desmin was investigated. Desmin plays an important role in organizing and maintaining the integrity and strength of the myofibrils and the overall cytoskeleton structure of the muscle fibre.[29] Degradation of this protein may therefore lead to some weakening of the myofibrillar structure. In agreement, the results showed that injection with actinidin-containing kiwifruit powder did induce desmin degradation.

Evidence for actinidin-induced changes in connective tissue at the ultrastructural level was obtained from atomic force microscopy of single muscle fibres. The images showed that actinidin induced damage to the endomysium. We speculate that actinidin degraded connective tissue structures that in turn have resulted in weakening of connective tissue and increased heat solubility of collagen. In agreement, hydrolysis of type I collagen by actinidin[10] and increased solubility of collagen after incubation of tendon with actinidin[8] have been reported. Furthermore, kiwifruit juice decreased the shear force of connective tissue and increased liberation of collagen-related peptides upon heat-treatment at 50 and 70 °C.[9]

The use of a brine solution containing actinidin or kiwifruit extract seems to have the potential to act as a tenderizer in tougher pork muscles without compromising taste and inducing a mushy unpleasant texture. Kiwifruit extract and actinidin has previously been reported to result in allergenic reactions.[30,31] However, a recent study has shown that actinidin is not a major allergen; instead, another protein with a molecular weight of approximately 38 kDa might cause allergenic reactions.[32] It is important to note that in the present study only two protein bands were clearly visible on the SDS-PAGE gel and the molecular weight of these bands was estimated to be around 25 and 23 kDa (Fig. 1). In accordance, purified actinidin was found to migrate at 25 kDa.[32]

In conclusion, injection of brine solution containing actinidin reduced WB shear force and improved sensory-assessed tenderness of cooked pork *biceps femoris*, whereas juiciness and flavour attributes were not affected. Analysis performed on myofibrils and connective tissue from raw meat showed increased degradation of desmin and percentage heat-soluble collagen at high levels of actinidin. Proteome analysis and myofibrillar fragmentation revealed no extensive degradation of muscle proteins following actinidin injection. AFM images of single muscle fibres showed actinidin-induced damage of endomysium.

ACKNOWLEDGEMENTS

The technical assistance of Julie Cecelia Stefansen and Ditte Ellegaard Christiansen from the University of Copenhagen as well as Ann-Britt Frøstrup and Mianne Darré from the Danish Meat Research Institute is gratefully acknowledged. This work was financed by the Directorate for Food, Fisheries and Agri Business (Denmark). Support for the work was also provided by the Danish Meat Research Institute (DMRI).

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