Food Sci. Biotechnol. 25(4): 1059-1064 (2016)
DOI 10.1007/s10068-016-0171-y

Food Science
and
Biotechnology

Isolation and characterization of a protease from the *Actinidia arguta* fruit for improving meat tenderness

Juan Wang[1,2], Haoming Liu[1], Haili Wang[1], Mingxun Cui[1], Qing Jin[1,2,3], Tie Jin[1,2], Fushun Cui[1,2,3], Taihua Cui[1,2], Chengyun Liang[1,2,3], Bumsik Kim[4], and Guanhao Li[1,2,3,*]

[1]*College of Agriculture, Yanbian University, Yanji 133002, Jilin, China*
[2]*Food Research Center, Yanbian University, Yanji 133002, Jilin, China*
[3]*Collaborative Innovation Center of Beef Cattle Science and Major Demand for Industrial Technology, Yanbian University, Yanji 133002, Jilin, China*
[4]*School of Food Science, Kyungil University, Gyeongsan, Gyeongbuk 38428, Korea*

Received January 17, 2016
Revised May 20, 2016
Accepted May 29, 2016
Published online August 31, 2016

*Corresponding Author
Tel: +86-433-243-5502
Fax: +86-433-243-5600
Email: ghli@ybu.edu.cn

pISSN 1226-7708
eISSN 2092-6456

Abstract An protease from *Actinidia arguta* for improving meat tenderness was purified, characterized from wild *A. arguta* fruit by ammonium sulfate precipitation, Sephdex G-25 gel filtration chromatography, and DEAE Sepharose Fast Flow ion exchange chromatography, and its activity was investigated. The purified protease was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis to obtain a single band of protease. The protease was purified successfully, and found to have a molecular weight of 23.8 kDa (mass spectrometry). The specific activity of the purified protease reached 53,428 U/mg with a 25.5-fold purification factor and 9% activity recovery. Based on N-terminal sequencing results, the *A. arguta* protease was derived from the class of actinidia proteases that have an N-terminal sequence of VLPDY VDWRS AGAVV. The protease was effective for tenderizing beef and decomposing actomyosin, suggesting the potential application for improving meat tenderness.

Keywords: plants, *Actinidia arguta*, protease, meat, tenderness

Introduction

Consumer acceptability of meat depends mainly on the tenderness of meat (1), and consumers prefer increased meat tenderness (2). Meat tenderness can be improved by hydrolyzing myofibrillar proteins (3). Plant proteases, as exogenous enzymes, have been used for improving meat tenderness because of their proteolytic activity. The applications of the plant proteolytic enzymes for improving meat tenderness are promising in the meat industry.

The most common proteolytic enzymes to tenderize meat included papain, ficin, and bromelain (4-6). These proteolytic enzymes can cause different degrees of hydrolysis of myofibrillar proteins. Despite the fact that plant proteases for improving tenderness are cheaply and commercially available, a need to discover new proteases from more plant sources because non-uniform or over activity of some proteases as a meat tenderizer still exists (7).

In a recent study, actinidin from *Actinidia chinensis* fruit (kiwifruit) that belongs to the Actinidiaceae family was used to tenderize meat (8). The proteolytic enzymes of kiwifruit were first discovered to have high levels of proteolytic activity in 1959 by Acrus (9). The actinidin protease was isolated, and its catalytic mechanism and basic properties were studied, including the molecular weight (Mw) of the enzyme, its activity center, and the methods of its extraction and purification (10-12). The activity center of the protease has an SH group, comprising 20 different amino acids, with a total of 169 amino acid residues (13). The commercial actinidin protease had been confirmed to be more effective at hydrolyzing beef myofibril proteins than other commercial plant proteases such as papain, bromelain, and zingibain (14), and is primarily used to tenderize meat.

A. arguta Planeh. is from the Actinidiaceae family as well. It is a perennial deciduous vine that produces a small fruit resembling the fruit of *A. chinensis*. This species is a wild plant that grows in Northern China, Korea, Japan, and Siberia. The fruit is rich in vitamins, minerals, and trace elements typically in *Actinidia* fruit. We found that the crude extract of *A. arguta* fruit has a desired tenderization effect on beef by improving the shear forces, cooking water loss, and water-holding capacity. However, tenderness effect of the protease from *A. arguta* fruit and its detailed report are lacking. Therefore, the article was aimed at isolating and characterizing a protease from *A. arguta* obtained from Changbai Mountain for improving meat tenderness and further assessing its activity.

Materials and Methods

Preparation of the crude extract Mature and wild *A. arguta* fruits were obtained from Changbai Mountain, Jilin Province, China. The *A. arguta* fruits were first washed in clean water and drained. Then, the drained *A. arguta* fruits were pressed in a juicing machine to extract their juice. Fifty milliliters of *A. arguta* fruit juice were mixed with 50 mL protease buffer containing 1 mM of EDTA and 0.1 M of L-cysteine at 4°C at pH 5.5 for 2 min. The mixed solutions were filtered through four layers of sterile gauze to remove any fibrous material. The crude filtrate was centrifuged at 4°C at 10,000× *g* for 20 min, and the supernatant (crude extract) was collected for further processing (15).

Beef tenderization test of the crude protease extract Beef (silverside of Yanbian yellow cattle) was obtained from a local market in Yanji City (Jilin Province, China). The meat samples (n=3) (3 cm^3 cubes) were soaked in 100 mL of 0.050% crude protease extract for 1 h at 50°C. The meat samples (n=3) (3 cm × 2 cm × 1 cm) were cut for the test of myofibrillar structure. The meat samples were fixed in formaldehyde solution, and then embedded and sliced. Myofibrillar density (n/mm^2) and diameter (μm) were observed by a photoelectric microscope (XSP-17C; Shanghai Changfang Optical Instrument Co., Ltd., Shanghai, China) with CF-2000XB image analysis software. The meat samples (n=3) (1 mm^3 cubes) were also used for myofibrillar ultrastructure test as following: the meat samples were placed in 3% glutaraldehyde immediately and fixed for 2 h. The samples were further fixed with 2% osmium tetroxide for 2-3 h. The samples were then washed with phosphate-buffered saline (PBS) and repeated three times. The samples were dehydrated using 30, 50, 70, 80, and 90% acetone for 30 min at each concentration, respectively. Then, the samples were dehydrated three more times with pure acetone (30 min each), and then embedded, polymerized, sliced, and double stained with uranyl acetate-lead citrate. The samples were finally photographed with a projection electron microscope (JEM-2100F; Hitachi, Tokyo, Japan).

Decomposition activity of actomyosin by enzyme The activity of enzyme for tenderizing meat may be predicted with actomyosin as a substrate (16). The actomyosin was extracted and isolated from fresh chicken breast following the methods reported by Rampton *et al.* (17) and Li *et al.* (18). In brief, actomyosin and protease were mixed with a ratio of 8:1 and reacted at pH 7.0 for 15 min with a reaction temperature of 60°C. Then, the sample (30 μL/well) was separated with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE contained a 12% (w/v) separating gel and an SDS electrophoresis buffer with 1.5 M Tris-HCl (pH 8.8). The process lasted for 2 h at ambient temperature. After electrophoresis, 10% of acetic acid in 30% methanol and 0.25% of Coomassie brilliant blue were used for stain and destain of the gel, respectively. Finally, the proteolytic activity of the enzyme was measured by a scan of the gel with an image scanner (Powerlook-1120; UMAX data system, Hsinchu, Taiwan).

Purification of protease Solid $(NH_4)_2SO_4$ was added to the crude extract to 70% saturation. The mixture was centrifuged (10,000× *g*, 4°C, 20 min) to remove the supernatant liquid. The precipitate was dissolved in 1 mM of EDTA (pH 8.0) at 4°C, and dialyzed against the same buffer solution for 24 h at 4°C. Then, the dialysate was replaced 3-4 times.

The dialyzed fluid was subjected to Sephdex G-25 gel column chromatography with a desalting column (XK26/40 Column with Sephadex G-25 Medium; column volume: 100 mL, flow rate: 10 mL/min). The column was connected to the AKTA purifier 100 system (GE, Fairfield, CT, USA). First, the protective solution (20% ethanol) was replaced with three times the column volume of distilled water. Then, five times the column volume of 0.1 M L-cysteine in 1 mM EDTA buffer (pH 6.5) was used for equilibration and elution of the column. The reaction of protease and actomyosin in elution was monitored by a Spectramax Plus^{384} (Molecular Devices, Sunnyvale, CA, USA) at 275 nm, and the active fraction was collected.

The active fraction was injected into the DEAE Fast Flow column (HiTrap DEAE FF; column volume: 1 mL, flow rate: 1 mL/min), and the solutions were equilibrated with 5 mL IEX buffer A (20 mM Na_3PO_4, pH 7.0); then, the column was washed with 5 mL IEX buffer A. The protease was eluted with the linear gradient elution method (19) (100% IEX buffer B in 20 min, ion exchange chromatography (IEX) buffer B: 20 mM Na_3PO_4, 1 M NaCl, pH 7.0). The active fraction was collected. The protein concentration was determined at 595 nm using the method with bovine serum albumin as the standard (20).

Determination of protease activity Casein was used as the substrate to determine the protease activity using UV spectroscopy (U-3900; Hitachi). One milliliter of protease solution (100 times of dilution) was mixed with 5.0 mL of casein solution (40 ppm). The mixture was incubated at 37°C for 10 min, and then, trichloroacetic acid solution (5 mL) was added to the mixture for 40 min. After filtration, the absorbance (A) of the filtrate was determined within 2 h by spectrophotometry at 275 nm with water as the blank. The casein solution and trichloroacetic acid were sequentially interchanged following the same procedure to determine the absorbance (A_0). A tyrosine control solution was used to determine the absorbance (A_n) at 275 nm with 0.1 M hydrochloric acid solution as the blank. The enzymatic activity was calculated as follows:

$$\text{Enzymatic activity (U/mg)} = \frac{A - A_0}{A_n} \times \frac{W_3}{W \times 10} \times 11 \times n$$

where W_3 is the amount of casein protein per 1 mL of control solution (μg); W is the sample size for the test (mg); 11 is the determined total volume; and n is the dilution factor of the sample.

The amount of protease required to produce 1 ìmol of tyrosine per minute by hydrolyzing casein was considered as 1 U of enzymatic activity.

Molecular weight determination and identification of the protease by mass spectrometry The sample (40 μL) was loaded onto 12% (w/v) polyacrylamide gel for the SDS electrophoresis at ambient temperature for 2.5 h (current intensity 20 mA). Afterwards, Coomassie brilliant blue and 30% methanol were used for stain and destain of the gel, respectively.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (4800 MALDI-TOF/TOF) (PerkinElmer, Waltham, MA, USA) was used to determine the molecular weight (Mw), and 4800 Plus MALDI TOF/TOF^{TM} Analyzer (GE) was used to identify the protease with the appropriate mass spectrometry database (data not shown). The significance was judged statistically by a probability (p) of 0.05.

After ZipTip C4 desalting treatment, 1 μL of the protein sample was spotted onto the sample spot and allowed to dry naturally. Then, 0.6 μL of sinapic acid (SA) solution was spotted onto the corresponding target, which was also allowed to dry naturally. The standard sample was spotted onto the targets adjacent to the samples in the same manner, and MALDI-TOF/TOF was then used to determine the Mw of the protease.

Gel bands from the SDS-PAGE were shredded and were added to 400 μL 100 mM NH_4HCO_3 in 30% acetonitrile (ACN). The sample was washed with 30 mM $K_3Fe(CN)_6$:100 mM $Na_2S_2O_3$=1:1 (v:v) to colorless and removed the supernatant. The sample was dehydrated after incubation with 100 mM NH_4HCO_3 at an ambient temperature for 15 min. The sample was dried after the removal of supernatant. The sample was incubated overnight with trypsin solution (trypsin/protein ration of 1:20) at 37°C. The sample was then incubated with 60% ACN in 0.1% trifluoroacetic acid (TFA) for 15 min. The sample was retained with digested peptides and ZipTip desalination, and then reconstituted with 2 μL of 20% ACN. The dissolved sample was dotted onto the sample target and allowed to dry naturally. Then, 0.5 μL of a supersaturated matrix solution containing 50% ACN and 0.1% TFA was dotted onto the corresponding target site, which was also allowed to dry naturally. The sample target was dried with a steady stream of nitrogen gas (99.999%) and then loaded into the instrument in the target slot for identification of the protease by MALDI-TOF/TOF.

Protease N-terminal sequencing After SDS-PAGE, the *A. arguta* protease was transferred onto a PVDF membrane (Millipore) by wet electrotransfer, stained with Coomassie brilliant blue, and then bleached. Two bands of samples and 1 mL of 0.1% TFA were added into tubes. The tubes were put into a vortex mixer for 30 s to mix the solution. The supernatant was then removed. The bands were cut into strips of 0.25 cm^2, and N-terminal sequencing was performed by an automatic protein sequencing instrument (PPSQ-33A; Shimadzu, Kyoto, Japan). The test conditions were as follows: pump flow of 1 mL/min, wavelength of 269 nm, and temperature of 48°C with 15 sample test cycles. A BLAST network service was performed to search the protein homology (21).

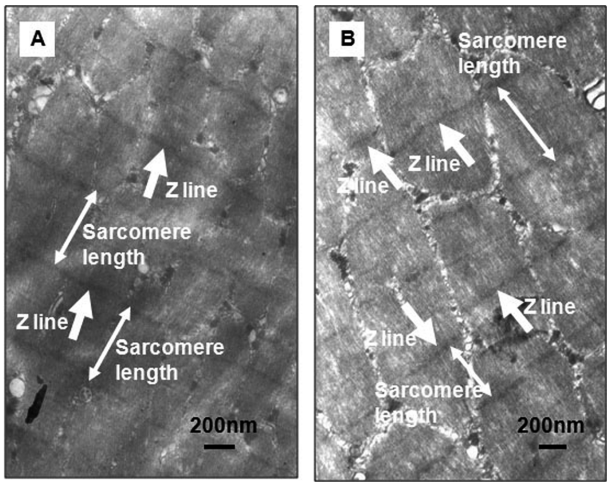

Fig. 1. Electron microscopy images of beef treated with the crude extract from *A. arguta* fruit compared with untreated beef (Bar=200 nm). A: control sample, B: treated sample.

Results and Discussion

Tenderization mechanism of protease from *A. arguta* fruit on meat To find the tenderization mechanism of protease from *A. arguta* fruit on meat, crude protease from *A. arguta* was extracted and tested in terms of its ability to tenderize beef. The tenderization effects of crude protease extract on beef were shown in Fig. 1. Unprocessed beef showed complete myofibrils, tight structure, uniform sarcomere length, and an intact Z line. In contrast, beef treated with the crude protease extract presented incomplete myofibrils, large myofibril gap, increased muscular interstition, non-uniform sarcomere length, and a Z line fracture. Moreover, the myofibrillar diameter of the treated beef decreased by 29.89% and the myofibrillar density increased by 24.32% (data not shown). These results indicated that the crude protease could effectively damage muscle structure by the hydrolysis effect of myofibrillar proteins to improve the tenderness. Tests of the shear forces, cooking water loss, and water-holding capacity also confirmed the improvement of beef tenderness (data not shown). Processed beef especially possesses good color and better taste.

The actomyosin is very suitable for the prediction of the tenderizing ability of the plant enzyme, which can hydrolyze the actomyosin to improve the tenderness of meat (9,10,22,23). The optimal stripe effect of crude protease extract for decomposing actomyosin (data not shown) revealed that the crude protease extract effectively decomposed actomyosin. The specific activity (2,098 U/mg) of the crude protease extract also revealed the strong proteolytic activity of the enzyme (Table 1). The results indicated that the crude protease contained the target protease with very good proteolytic activity on actomyosin. In later experiments, purification of enzyme was performed on the basis of proteolytic activity on actomyosin.

Table 1. Purification data of protease from *A. arguta* fruit (*n*=3)

Purification step	Protein content (mg)	Enzyme activity (U)	Specific activity (U/mg)	Purification (fold)	Activity recovery (%)
Crude protease extract	100.96	2.1×10^5	2,098	1.0	100
Dialysis	19.40	1.5×10^5	7,530	3.6	71
Sephdex G-25 column chromatography	4.49	5.6×10^4	12,574	6.0	27
DEAE FF ion exchange chromatography	0.35	1.9×10^4	53,428	25.5	9

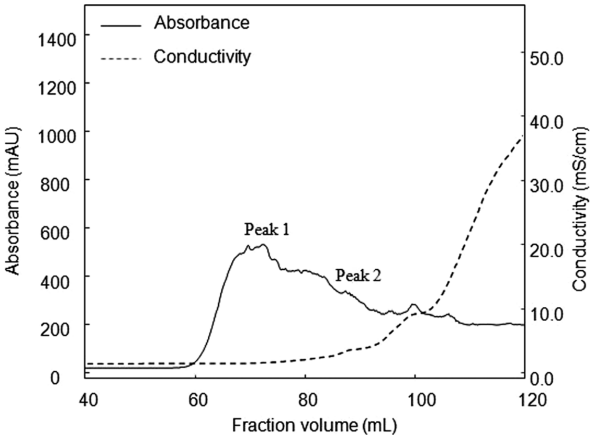

Fig. 2. Elution profile of the protease from *A. arguta* after purification by Sephdex G-25 column chromatography (Sephdex G-25 Medium, XK26/40 Column). Elution buffer: 500 mL cysteine buffer dilution, flow rate: 10 mL/min, and column volume: 100 mL.

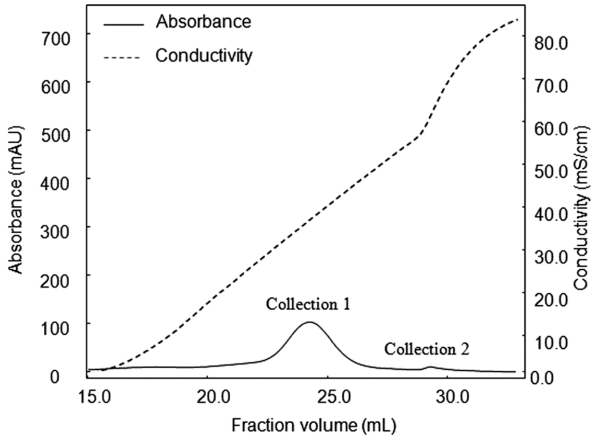

Fig. 3. Elution profile of the protease from *A. arguta* after purification by DEAE FF ion exchange chromatography (HiTrap DEAE FF). Elution buffer: IEX buffer A (20 mM Na_3PO_4, pH 7.0), gradient: IEX buffer B (20 mM Na_3PO_4, 1 M NaCl, pH 7.0) from 0% to 100% in 20 min. Flow rate: 1 mL/min and column volume: 1 mL.

Purification and activity of proteolytic enzyme To improve the ability of the protease to tenderize the meat, *A. arguta* protease was isolated from the crude protease. Table 1 displays the purification scheme. In the purification scheme, ammonium sulfate precipitation was used to concentrate and purify the protein from the crude protease extract. The protein content (19.40 mg) and activity recovery (71%) from the dialysis after ammonium sulfate precipitation were reduced with increasing purification (3.6-fold) and specific activity (7,530 U/mg) (Table 1). Some non-active proteins were removed from the crude protease extract. Sephdex G-25 gel filtration chromatography separated proteins with different relative Mw. Sephdex G-25 gel filtration chromatography of the crude protease extract revealed two main proteolytic peaks: fraction 1 (containing the *A. arguta* protease) and fraction 2 (Fig. 2). Fractions 1 and 2 were collected to test their ability to decompose actomyosin (data not shown), and both were able to decompose actomyosin. However, fraction 1 had better efficacy and thus was selected for further purification. The protein content (4.49 mg) and activity recovery (27%) of fraction 1 (containing the *A. arguta* protease) were further reduced with increasing purification (6.0-fold) and specific activity (12,574 U/mg) (Table 1). Sephdex G-25 gel filtration chromatography removed additional nonenzymatic proteins. Subsequently, DEAE FF ion-exchange chromatography was used to separate and purify proteins with the differences in the charge of proteins. DEAE FF ion-exchange chromatography yielded two main proteolytic peaks (Fig. 3), and collection 1 was more effective for actomyosin decomposition (*A. arguta* protease) (data not shown). The results of actomyosin decomposition indicate that the *A. arguta* protease presents good potency for tenderizing meat. In addition, a 25.5-fold degree of purification and 0.35 mg of protein content were obtained for the *A. arguta* protease (collection 1) (Table 1). The specific enzymatic activity reached 53,428 U/mg and the activity recovery was 9% (Table 1). These results reveal excellent purification with high specific activity.

Molecular weight The SDS-PAGE revealed that the purified *A. arguta* protease appeared as a unique band with a relative Mw of 25 kDa (Fig. 4). The Mw of the *A. arguta* protease (23.8 kDa) was then confirmed by mass spectroscopy (Fig. 5). The results were similar with those obtained from other *Actinidia* proteases. Bolank and Herdnan (10) reported an *Actinidia* protease with Mw of 26 kDa, whereas Carne and Moore (11) found an *Actinidia* protease with Mw of 23.5 kDa. Further, the *Actinidia* protease from *A. chinensis* was shown to have Mw of 20.89 kDa (13), and the *Actinidia* proteases isolated from *Actinidia chinensis* Planch. had Mw ranging from 23.5 to 24.0 kDa (24).

Protein sequence Ten protein dots were analyzed by mass spectrometry and all proteins could be retrieved from the database (data

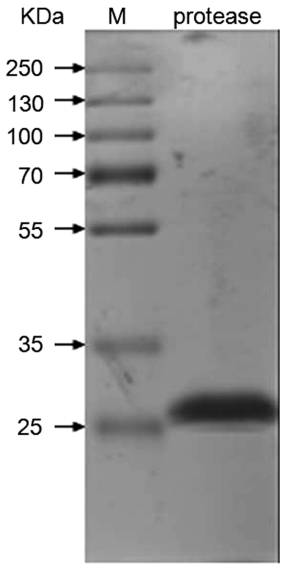

Fig. 4. SDS-PAGE electrophoretogram of the protease purified from *A. arguta* fruit.

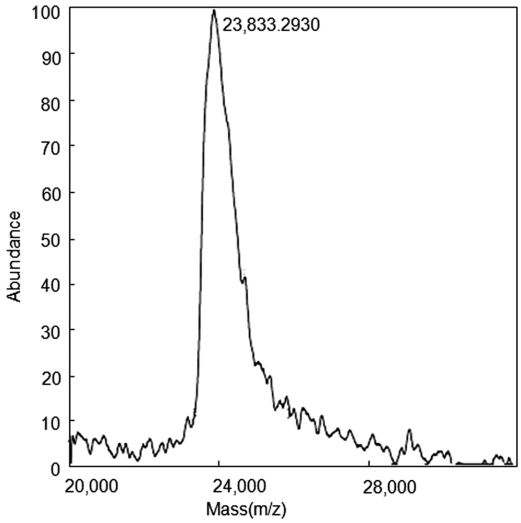

Fig. 5. Mass spectrogram of the *A. arguta* protease.

not shown). The *A. arguta* protease was successfully identified (matched marks >60 and p<5%). Proteins 1 and 2 were actinidin Act1b and Chain A, respectively.

The electrotransfer spectrum of the *A. arguta* protease was analyzed (data not shown). The single band met the purity requirements for N-terminal sequencing mass spectrometry. The N-terminal sequencing of the *A. arguta* protease revealed a sequence of 15 amino acids (data not shown): VLPDY VDWRS AGAVV. The online software BLAST in the database NCBInr was used to obtain the amino acid sequence for homology comparison (25). The E value was 1e-06, the identities were 100%, positives were 100%, and gaps were 0%, showing 100% homology of the N-terminal sequence of the *A. arguta* protease and the actinidin Act1b. The sequence matched the 126th amino acid in the sequence of the actinidin Act1b and lacked the first 125 amino acids. The results reveal that the *A. arguta* protease extracted in this study is an *Actinidia* protease with an N-terminal sequence of VLPDY VDWRS AGAVV.

Acknowledgments This study was supported by a grant from the National Natural Science Foundation of China (No. 31160327).

Disclosure The authors declare no conflict of interest.

References

1. Huffman KL, Miller MF, Hoover LC, Wu CK, Briton HC, Ramsey CB. Effect of beef tenderness on consumer satisfaction with steaks consumed in the home and restaurant. J. Anim. Sci. 74: 91-97 (1996)
2. Marsh BB, Carse WA. Meat tenderness and sliding-filament hypothesis. Int. J. Food Sci. Tech. 9: 129-139 (1974)
3. Savell JW, Cross HR. Animal product options in the market place. pp. 345-355. In: The role of fat in the palatability of beef, pork, and lamb. Council NR (ed). National Academy Press, Washington DC, USA (1988)
4. Brooks BA, Kalsing KC, Regenstein JM. Effects of antemortem injected crude papain in chicken muscle. J. Food Sci. 50: 1370-1375 (1985)
5. Kee HJ, Hwang YS, Kim KH, Hog YH. Application fig protease to foods. Korean J. Food Sci. An. 18: 19-26 (1998)
6. Lee DH, Jin BH. Encapsulation of bromelain in liposome. J. Food Sci. Nutr. 5: 81-85 (2001)
7. Kim EM, Choe LS, Hwang SG. Effects of singular or mixed type treatment of proteases isolated from pear, pineapple and kiwifruit on actomyosin degradation. Korean J. Food Sci. An. 23: 193-199 (2003)
8. Rødbotten R, Solgaard K, Johannessen TC. Effect of kiwi fruit powder in tenderizing *M. Triceps brachii* from mature cows. Meat Sci. 101: 117 (2015)
9. Arcus AC. Proteolylic enzymes of *Actinidia chinensis*. Biochim. Biophys. Acta 33: 241-243 (1959)
10. Bolank MJ, Herdnan MJ. Kinetic studies on the thiol protease from *Actinidia chinensis*. FEBS Lett. 27: 251-254 (1972)
11. Carne A, Moore CH. The amino acid sequence of the tryptic peptides from Actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. J. Biochem. 173: 73-78 (1978)
12. Brocklehurst K, Baines BS, Malthouse JPG. Differences in the interactions of the catalytic groups of the active centers of actinidin and papain. J. Biochem. 197: 739-741 (1981)
13. Edward N, Baker MJ, Boland PC. The specificity of actinidin and its relationship to the structure of the enzyme. BBA - Enzymology 616: 30-34 (1980)
14. Ha M, Bekhit AA, Carne A, Hopkins DL. Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. Food Chem. 134: 95-105 (2012)
15. Kim JY, Lee IK, Son MW. Effects of orally administered Actinidia arguta fruit extract on 2-chloro-1,3,5-trinitrobenzene-induced atopic dermatitis like skin lesions in NC/Nga mice. J. Med. Food 12: 1004-1015 (2009)
16. Weber K, Osborn M. The reliability of molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412 (1969)
17. Rampton JH, Pearson AM, Walker JE, Kapsalis JG. Disc electrophoresis of Weber-Edsall extract and actomyosin from skeletal muscle. J. Agr. Food Chem. 19: 238-240 (1971)
18. Li GH, Mandal PK, Lim H, Baatartsogt O, Chi HL, Jeon SC. Choi K. Purification and characterization of a protease from Korean pear (*Pyrus serotina L*) as meat tenderizer. Korean J. Food Sci. An. 29: 157-163 (2009)
19. Kyung HC. Amino-acid sequence and glycan structures of cysteine proteases with proline specificity from ginger rhizome Zingiber off icinale. Eur. J. Biochem. 267: 1516-1526 (2000)
20. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. Anal. Biochem. 72: 248-254 (1976)
21. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSIBLAST: A new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402 (1997)
22. Miyada DS, Tappel AL. The hydrolysis of beef proteins by various proteolytic enzymes. Food Res. Int. 21: 217-223 (1956)
23. Kang CK, Rice EE. Degradation of various meat fractions by tenderizing enzymes. J. Food Sci. 35: 563-567 (1970)
24. Xiang D, Zhang J, Chen Y. Expressions and purification of a mature form of

recombinant human chemerin in escherichia coli. Protein Expres. Purif. 69: 153-158 (2010)
25. Nieuwenhuizen N, Beuning L, Sutherland P, Sharma N, Cooney J, Bieleski L, Schroeder R, MacRae E, Atkinson R. Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit Funct. J. Plant Biol. 34: 946-961 (2007)