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Purification and Characterization of a Protease from Korean Pear (*Pyrus serotina* L.) as Meat Tenderizer

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Abstract

This study was conducted for the isolation, purification, and characterization of a protease from Korean pear, to see its proteolytic activity on chicken actomyosin and to find the optimum pH and temperature of activity on chicken actomyosin. The protease was isolated from crude extract of Korean pear by ammonium sulfate precipitation. Further purification was done by DEAE-Sepharose ion-exchange chromatography, Mono-Q and Mini-Q column chromatography. The purified enzyme gave a single protein band on SDS polyacrylamide gel electrophoresis and the molecular weight was found to be 38 kDa. The specific activity of purified enzyme was 34,907 unit/mg with 25 fold purification and the yield was 2%. The purified enzyme incubated with chicken actomyosin showed high activity. The optimum pH and temperature for enzyme activity on chicken actomyosin were 6.5 and 70°C, respectively. A protease was purified from Korean pear for the first time and characterized. It was found to be promising for meat tenderization.

Key words : Korean pear protease, characterization, proteolytic activity, chicken actomyosin, tenderizer

Introduction

Tenderness has been identified as the most important factor for consumer acceptability of meat. Meat tenderness is strongly influenced by a sliding-filament mechanism of actin over myosin and actomyosin is a major contributor of toughness (Marsh and Carse, 1974). The researches on the use of proteolytic enzymes in meat tenderization have been actively underway (Blanchard and Mantle, 1996). Most of the proteolytic enzymes of plant origin have been shown to increase the tenderness of meat by the hydrolysis of myofibrillar proteins mainly on actomyosin (Miyada and Tappel, 1956; Kang and Rice, 1970).

The proteolytic enzymes of plant origin extensively studied and most commonly applied for meat tenderization include papain from papaya (Brooks *et al.*, 1985), ficin from figs (Kee *et al.*, 1998), bromelain from pineapples (Lee and Jin, 2001). More recent studies are application of zingibain from ginger (Naveena and Mendiratta, 2001; Naveena *et al.*, 2004) actinidin from kiwifruit (Roh *et al.*, 2002; Nam *et al.*, 2006), cucumin from cucumis (Naveena *et al*, 2004), and protease from mashroom (*Sarcodon aspratus*) (Lee and Jang, 2005). They are widely reported on their chemical properties on many research papers. But the practical limitation with the enzymes such as ficin, bromelanin and papain is their non-uniform or over activity and thus sometime lower the commercial value of the meat (Kim *et al.*, 2003).

The proteolytic properties of crude extract from Korean pear and its activity on chicken myosin with optimum pH (5.3-7.0) and temperature (40°C) has been reported (Choe and Park 1996). Another study reported (Choe *et al.*, 1996) on the activity of crude protease extracted from Korean pear on chicken actomyosin for tenderization. The tenderizing ability of crude protease from pear individually or in combination with pineapple or kiwifruit extract on chicken actomyosin was further emphasized (Kim *et al.*, 2003). A recent study (Han and Chin, 2004) partially purified protease from Korean pear and suggested approximate molecular weight (30 kDa) of the

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protease and the optimum concentration (0.1%) for tenderization in pork loins. However, no report is available on the complete purification, characterization and activity of the protease from Korean pear.

Therefore, the present study was undertaken for isolation, purification and characterization of protease from Korean pear. Furthermore its proteolytic activity on chicken actomyosin and meat tenderization ability were examined for wide application in meat industry which may open new economic utilization for pear

Materials and Methods

Extraction and Isolation of protease

The method of Choe *et al.* (1996) was slightly modified to extract proteolytic enzymes from Korean pear. Fresh pear (Shingo breed) was procured from Anseong city, Gyeonggi province, peeled after cleaning, cut into pieces, ground and filtered through cheese cloth to get the juice. Equal parts of fruit juice and 0.1 M L-cysteine in 1 mM EDTA (pH 6.5) solutions were mixed and homogenized at 4°C for 2 min. Then, the solution was filtered through a sterilized gauze and centrifuged (Avanti^R J-E, Beckman Coulter, USA) at 4°C for 20 min at 10,000×*g*. The supernatant was mixed with 0.1 M L-cysteine in 1 mM EDTA (pH 7.0) solution, followed by dialysis for 24 h in the solutions of pH 6.5 and used as the crude extract. The crude extract was mixed with 70% ammonium sulfate solution to precipitate the proteins and precipitate was kept at -70°C till further use. The proteolytic enzyme was isolated by dissolving the precipitate in the solution of 0.1 M L-cysteine in 1 mM EDTA (pH 6.5) and dialyzing the solution with 0.45 μ membrane filter at 4°C for 12 h.

Purification of protease

DEAE Sepharose ion-exchange chromatography

The protease isolated from crude extract by ammonium sulphate precipitation and dialysis was used for chromatography as per standard procedure. Sample was injected to DEAE-Sepharose column (Amarsham Bioscience) at a rate of 0.5 mL/min as 1 mL/tube and proteolytic enzyme was eluted in 0.5 M NaCl. Pooled fractions containing activity of protease from the first DEAE-Sepharose chromatography was equilibrated with 0.1 M L-cysteine in 1 mM EDTA buffer (pH 6.5). The protease was eluted with 0 M to 1 M NaCl gradient using 0.1 M L-cysteine, 1 mM EDTA buffer (pH 6.5). The reaction of protease in the sample on chicken actomyosin was measured at 280 nm using Spectramax Plus^{384} (Molecular Devices Corp, USA).

Mono-Q column chromatography

The fractions eluted and fractionated by DEAE-Sepharose chromatography were filtered with membrane filter (Millipore, YM 30, USA) followed by secondary purification and fractionation with Mono-Q column of 1 mL capacity (AKTA Basic10, Amersham Pharmacia Biotech, USA) at the rate of 0.5 mL/min as 1 mL/tube. Activity of protease from pooled fractions from the Mono-Q column was measured as described earlier in case of DEAE chromatography.

Mini-Q column chromatography

The fractions eluted and fractionated by Mono-Q chromatography were filtered with membrane filter (Millipore, YM 30, USA) followed by final purification and fractionation with Mini-Q column of 0.5 mL capacity (C18 Microspin column, Vivascience, Germany) at the rate of 0.5 mL/min as 0.5 mL/tube. Activity of protease from pooled fractions from the Mini-Q column was measured as described incase of DEAE chromatography.

Extraction and isolation of actomyosin

The method of Rampton *et al.* (1971) was slightly modified to extract actomyosin. Chicken breast muscle was cut into thin strips and mixed with three parts of Weber solution (0.6 M KCl) and stored at 4°C for 24 h. Then two parts (w/v) of 0.6 M KCl solution were added and mixed thoroughly. The mixture was centrifuged at 4°C for 1 h at 15,000×*g*, the supernatant was filtered through a sterilized gauze at about 4°C and mixed with an equal part of distilled water for 5 min. Then the filtrate was centrifuged for 10 min at 4°C at 10,000×*g* and the precipitate was collected. The precipitate was mixed with an equal part of 1 M KCl solution and centrifuged for 1 h at 4°C at 15,000×*g*. About three parts of distilled water was added to the supernatant and again centrifuged for 10 min at 4°C at 10,000×*g*. The precipitate was then dissolved in 1 M of KCl solution, dialyzed in 0.6 M NaCl solution for 12 h, and centrifuged for 1 h at 4°C at 15,000×*g*. The supernatant was then mixed with equal part of glycerin and stored at -70°C (Choe *et al.*, 1996).

Protein estimation

The protein content in the solution at different stage of

purification of enzyme and in the actomyosin solution was estimated using biuret method (Gornall *et al.*, 1948). Bovine serum albumin (Bio-Rad, USA) was used as standard and OD was measured by a spectrophotometer (Spectramax Plus^{384}, Molecular Devices Corp, USA). The protein content in the eluted fraction after reaction with actomyosin was measured (Bradford, 1976) using Bio-Rad protein assay kit (Bio-Rad, USA) and OD was measured by the spectrophotometer.

Measuring enzyme activity

Mixture of actomyosin and protease at the ratio of 10:1 was reacted at 25°C for 1 h and 1 mg/mL sample was produced. Sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done following the method of Laemmli (1970) using an electrophoresis apparatus (Ettan IPG Phor-II, Amersham Biosciences). The sample was loaded at 30 μL/well and run for 2 h at room temperature by using 12% (w/v) polyacrylamide gels with the SDS electrophoresis buffer containing 1.5 M Tris-HCl (pH 8.8). After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant and destained with 10% acetic acid in 30% methanol. The gel was scanned through image scanner (UMAX data system, Powerlook-1120, Taiwan) and Sigma Gel Software (Jandel Scientific Co., USA) was used to verify the amount of digestion of actomyosin by protease. The amount of enzyme needed to dissolve 1 μg of actomyosin was set to 1 unit.

Measuring molecular weight

Electrophoresis (SDS-PAGE) of the purified enzyme was performed according to Weber and Osborn (1969) using Ettan IPG Phor-II (Amersham Biosciences) for measuring molecular weight. The sample was loaded at 30 μL/well on 12% (w/v) polyacrylamide gels and stained by silver staining. From the movement of the proteins in marker and sample in the gel an electrophoretic mobility plot was made and molecular weight of the sample protein was obtained.

Effect of pH on the activity of protease

To study the effect of pH on the enzyme activity of protease on chicken actomyosin, 50 μL protease purified from Korean pear was incubated with 100 μL of actomyosin solution (2.03 mg/mL) at 37°C using 0.1 M L-cysteine, 1 mM EDTA buffer at different pH between 3.0 to 9.0 with 0.5 interval. The proteolytic activity in each case was measured separately through SDS-PAGE.

Effect of temperature on the activity of protease

To study the effect of temperature on enzyme activity of protease on chicken actomyosin, 50 μL protease purified from Korean pear was incubated with 100 μL of actomyosin solution (2.03 mg/mL) using 0.1 M L-cysteine, 1 mM EDTA buffer with pH 6.5 at the temperature of 4, 25, 37, 60, 70 or 80°C. The proteolytic activity in each case was measured separately through SDS-PAGE.

Measuring proteolysis by SDS-PAGE

The weight ratio between actomyosin and protease was set to 1:1. Electrophoresis (SDS-PAGE) was done as reported by Laemmli (1970). The sample was loaded at 30 μL/well and run for 2 h at room temperature by using 12% (w/v) polyacrylamide gels with the SDS electrophoresis buffer containing 1.5 M Tris-HCl (pH 8.8). After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant and destained with 10% acetic acid in 30% methanol. The gel was scanned through image scanner (UMAX data system, Powerlook-1120, Taiwan) and Sigma Gel Software (Jandel Scientific Co., USA) was used to verify the amount of digestion of actomyosin by protease.

Results and Discussion

Purification of proteolytic enzyme

A protease was purified from Korean pear through ammonium sulphate precipitation, DEAE sepharose, Mono-Q and Mini-Q chromatography. The yield, fold of purification and specific activity of the protease from pear at different stage of purification is presented in Table 1.

The specific activity of the crude extract obtained from Korean pear was found to be 1,396 unit/mg. The specific activity of the enzyme indicated the satisfactory proteolytic activity of the enzyme on actomyosin. The yield (36%) of the enzyme from the dialysis after ammonium sulfate precipitation was reduced with marginal improvement in purification (1.2 fold) and specific activity (1,631 unit/mg). The reduced yield may be due to the presence of substantial amount of non-active proteins present with the crude extract which the ammonium sulfate precipitation could not remove. The result showed 2 peaks (Fig. 1) in the elution and fractionation of protein after DEAE sepharose chromatography. Fraction 18, 19 and 20 were found to be active and were collected as single fraction. The yield (16%) was further

Table 1. Purification data, specific activity and yield of protease from Korean pear

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification fold
1. Crude extract	130.59	182,350	1,396	100	1.0
2. Ammonium sulfate precipitation	40.12	65,430	1,631	36	1.2
3. DEAE-Sepharose chromatography	7.80	30,019	3,849	16	2.8
4. Mono-Q chromatography	1.11	11,013	9,922	6	7.1
5. Mini-Q chromatography	0.14	4,887	34,907	2	25.0

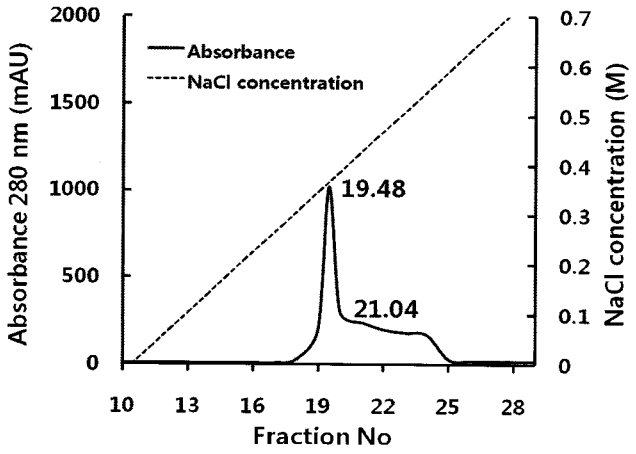

Fig. 1. Elution profile of protease from Korean pear purified with DEAE Sepharose chromatography. Protease from the first DEAE-Sepharose chromatography was equilibrated with 0.1 M L-cysteine in 1 mM EDTA buffer (pH 6.5). The protease was eluted with 0 to 1 M NaCl gradient using 0.1 M L-cysteine, 1 mM EDTA buffer (pH 6.5). The reaction of protease in the sample on chicken actomyosin was measured at 280 nm.

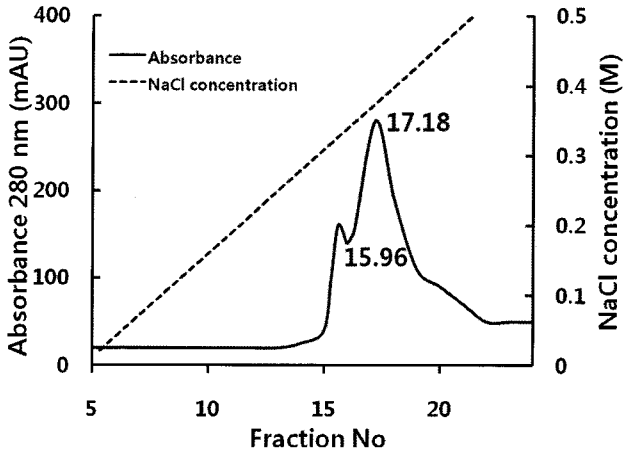

Fig. 2. Elution profile of protease from Korean pear purified with Mono-Q chromatography. Secondary purification and fractionation with Mono-Q column of 1 mL capacity (AKTA Basic10, Amersham Pharmacia Biotech, USA) at the rate of 0.5 mL/min as 1 mL/tube. Activity of protease from pooled fractions from the Mono-Q column was measured at 280 nm.

reduced with some improvement in purification (2.8 fold) and activity (3,849 unit/mg). The lower specific activity indicated the inefficiency of DEAE chromatography which could remove the nonenzymatic proteins partially.

There were 2 peaks from Mono-Q chromatography and the elution of proteolytic enzyme was at fraction 17 and 18 (Fig. 2). The yield was 6% with 7.1 fold purification and 9,922 unit/mg of specific activity. The heavy decrease in yield indicates lower efficiency of the Mono-Q column in removing the nonenzymatic proteins, however, there was good improvement in purification as indicated by significant increase in specific activity. A single peak (Fig 3) obtained from Mini-Q column after filtration with membrane filter. This indicated considerable removal of other impure protein which had not been absorbed in Mono-Q column. The result has shown 2% of yield with 25 fold purity and 34,907 unit/mg of specific activity. The Mini-Q chromatography has given very good purification as indicated by very high specific activity but the yield was found to be very low, which may be a limitation for its commercial application.

Enzyme activity

The purified enzyme was found to have very good proteolytic activity on chicken actomyosin as indicated by the specific activity of the protease at different stage of purification. Most of the protease of plant origin have been reported to hydrolyze myofibrillar protein (Kang and Rice, 1970) and it was logical to use actomyosin as a substrate to predict the tenderizing potency of the enzyme which was also suggested in earlier report (Weber and Osborn, 1969). Therefore, it can be inferred that the protease isolated from Korean pear should be suitable for meat tenderization which was already reported in some earlier studies (Choe and Park, 1996; Choe *et al.*, 1996; Han and Chin, 2004).

Molecular weight

SDS-PAGE electrophoresis of the purified enzyme has shown a single band and the molecular weight was mea-

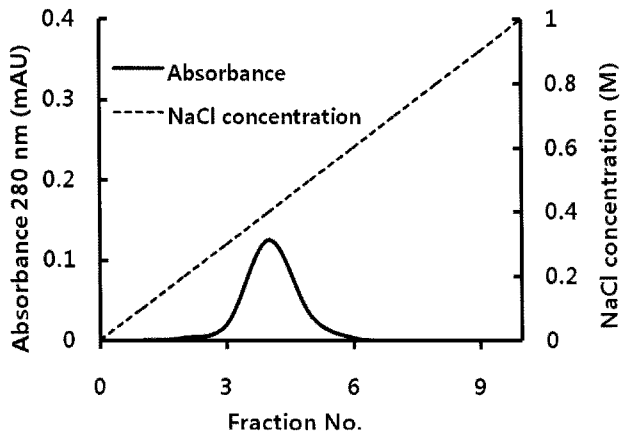

Fig. 3. Elution profile of protease from Korean pear purified with Mini-Q column. Final purification and fractionation with Mini-Q column of 0.5 mL capacity (C18 Microspin column, Vivascience, Germany) at the rate of 0.5 mL/min as 0.5 mL/tube. Activity of protease from pooled fractions from the Mini-Q column was measured at 280 nm.

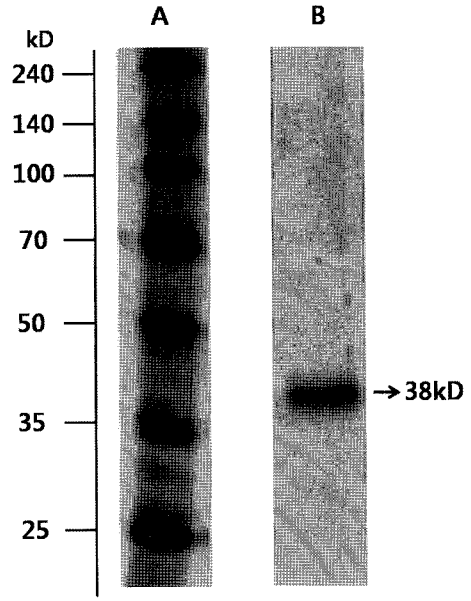

Fig. 4. SDS-PAGE of protease purified from Korean pear (A: Marker; B: Sample). The sample was loaded at 30 μL/well on 12% (w/v) polyacrylamide gels and stained by silver staining. From the movement of the proteins in marker and sample in the gel an electrophoretic mobility plot was made and molecular weight of the sample protein was obtained.

sured to be 38 kDa (Fig. 4). Han and Chin (2004) reported an approximate molecular weight of partially purified protease from Korean pear as 30 kDa. This difference is clearly due to imperfect measurement of molecular weight from partial purification of enzyme in their study. However, in the present study enzyme was perfectly purified through several stages and it is evident by the single band in electrophoresis.

Effect of pH on enzyme activity

Degradation activity of proteolytic enzyme purified from pear has shown a pH range of 3.0-7.5 for stable activity with an optimum pH of 6.5 (Fig. 5). This is in consistent with the earlier report of Choe *et al.* (1996) which stated maximum degradation of chicken myosin at pH 5.5 and 7.0. Kim *et al.* (2003) observed active degradation activity of pear extract on chicken actomyosin at pH 5.3 and 7.0 and little activity at pH 8.0. Yang *et al.* (1975) also concluded that the maximum activity of protease in commercial meat tenderizer was at pH 6-7. The optimum pH of the proteolytic enzyme purified in this study is suitable for tenderization of meat (Naveena *et al.* 2004).

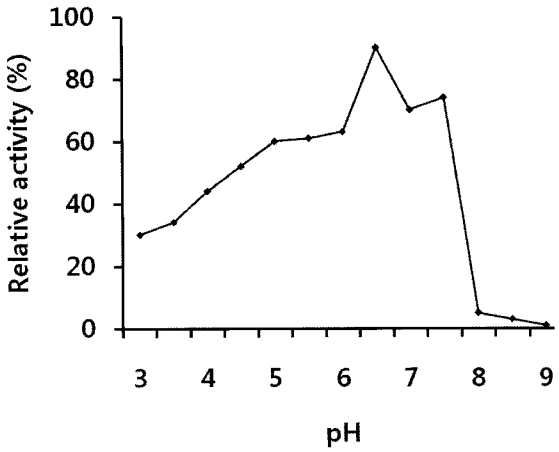

Fig. 5. Effect of pH on the activity of protease purified from Korean pear on chicken actomyosin. Fifty μL protease purified from Korean pear was incubated with 100 μL of actomyosin solution (2.03 mg/mL) at 37°C using 0.1 M L-cysteine, 1 mM EDTA buffer at different pH between 3.0 to 9.0 with 0.5 interval. The proteolytic activity in each case was measured separately through SDS-PAGE.

Effect of temperature on enzyme activity

The proteolytic activity of the enzyme purified from pear on actomyosin at pH 6.5 has shown progressively increased activity from 37-70°C and sharp fall above 70°C, hence the optimum temperature was 70°C (Fig. 6). Yang *et al.* (1975) has also reported optimum temperature for proteolytic activity of commercial meat tenderizer as 60-70°C. However, Choe and Park (1996) have reported stable activity of the crude extract of pear up to 50°C with a maximum activity at 40°C on chemical substrate (CBZ-lys-Onp) at pH 7.0. This difference

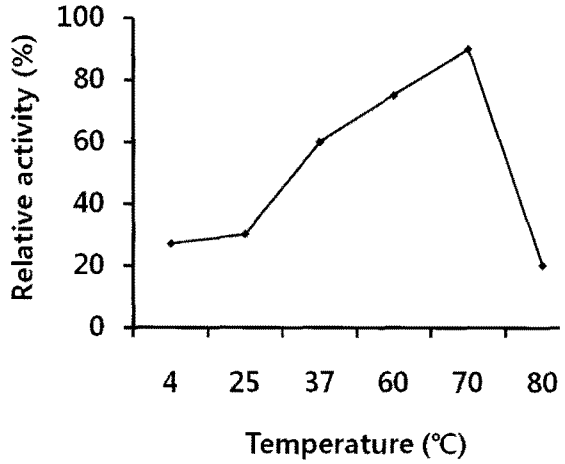

Fig. 6. Effect of temperature on the activity of protease purified from Korean pear on chicken actomyosin. Fifty μL protease purified from Korean pear was incubated with 100 μL of actomyosin solution (2.03 mg/mL) using 0.1 M L-cysteine, 1 mM EDTA buffer with pH 6.5 at the temperature of 4, 25, 37, 60, 70, 80°C. The proteolytic activity in each case was measured separately through SDS-PAGE.

may be due to the use of chemical substrate and neutral pH.

Conclusion

In conclusion, a proteolytic enzyme was successfully purified and characterized for the first time from Korean pear using different stage of purification. The enzyme was found to have very good proteolytic activity on chicken actomyosin and it is expected to be very promising because of its optimum pH (6.5) and temperature (70°C) is ideal for meat tenderization.

Acknowledgements

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