J Appl Biol Chem (2015) 58(2), 97–100
http://dx.doi.org/10.3839/jabc.2015.016

Online ISSN 2234-7941
Print ISSN 1976-0442

Original Article: Biochemistry/Molecular Biology

Characterization of the Intact Form of *Thermotoga maritima* Pectinase TmPecN Expressed in *Escherichia coli*

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Received: 20 August 2014 / Accepted: 11 September 2014 / Published Online: 30 June 2015

Abstract The thermostable pectinase gene *TmPec* isolated from *Thermotoga maritima* was introduced into the *Nde*I site of pRSET-B vector and expressed in its intact form in *Escherichia coli* BL21. The overexpressed intact form of pectinase (TmPecN protein) was partially purified by heat-denaturation procedure. TmPecN showed the highest activity between 85 and 95°C, and at approximately pH 6.5. Enzyme activity was stably maintained at temperatures below 85°C. In the presence of Ca^{2+}, pectinase activity of TmPecN increased to 128.4% of normal level. In contrast, Ba^{2+}, Zn^{2+}, and Mn^{2+} strongly inhibited TmPecN activity. We conclude that the biochemical properties of the intact form of TmPecN are comparable to those of the recombinant protein TmPec reported previously.

Keywords heat-denaturation · pectin · pectinase · thermostable · *Thermotoga maritima*

Introduction

Pectins are composed of a homogalacturonan backbone and highly branched rhamnogalacturonan. They constitute the plant cell wall matrix, holding cellulose and hemicellulose fibers together (Carpita and Gibeaut, 1993). Enzymatic degradation of pectins is a critical processing step for food, textile and paper processing industries (Kashyap et al., 2001).

Pectinases, a general term for pectin-hydrolyzing enzymes, are classified into polygalacturonases, pectin esterases, pectin lyases, and pectate lyases, depending on their mode of action (Alkorta et al., 1998). Pectinases have been isolated from a wide range of microorganisms, including bacteria (Dosanjh and Hoondal, 1996; Kapoor et al., 2000; Kashyap et al., 2000), yeast (Blanco et al., 1999), fungi (Stratilova et al., 1996; Huang and Mahoney, 1999), and *Actinomycetes* (Bruhlmann, 1995; Beg et al., 2000). The enzymes are also isolated from mesophilic fungi and bacteria, for use in various industries. Recently, a few thermostable pectate lyases have been isolated and characterized, and have subsequently attracted industrial attention (Kozianowski et al., 1997; Takao et al., 2000).

Hyperthermophilic microorganisms are of considerable industrial interest because they are natural sources of heat-stable enzymes useful in biotechnology. *Thermotoga maritima* grows optimally at 80°C (maximal growth at 90°C), and the genus represents one of the most thermophilic eubacterial genera yet identified (Huber et al., 1986). *T. maritima* metabolizes a variety of polysaccharides, such as xylan, starch and cellulose (Huber et al., 1986; Chhabra et al., 2002). This family of microbes facilitates the isolation of highly thermostable enzymes that can be used as valuable catalysts for industrial purposes.

Previously, we inserted the *T. maritima* pectinase gene *TmPec* into an *Eco*RI site of the pRSET expression vector to fuse with a histidine tag, and characterized the biochemical properties of the purified recombinant TmPec protein (Kim, 2014). In the present study, the *TmPec* gene was cloned into an *Nde*I site of the pRSET vector to be expressed in its intact form. The intact form TmPecN protein was partially purified by heat-denaturation, and its biochemical properties were analyzed.

Materials and Methods

Bacterial strains. Chromosomal DNA from *T. maritima* (ATCC

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43589D) was purchased from ATCC and was used to clone *TmPec*. *E. coli* TOP10 was used for transformation and plasmid propagation, and *E. coli* BL21 for *TmPec* expression.

Enzymes and reagents. Restriction enzymes and DNA-modifying enzymes were purchased from Promega Inc. (USA). All enzymes were used according to manufacturers' instructions. Pectin and other reagents were purchased from Sigma Chemical Co. (USA).

Construction of expression plasmid. The open reading frame (ORF) of the *TmPec* gene excluding a putative signal sequence encoding 27 amino acids was introduced into the pRSET-B expression vector by polymerase chain reaction (PCR) and overexpressed in *E. coli* BL21. The *TmPec*-specific primers used were 5'-AAATTTCATATGTCTCTCAATGACAAACCT-3' and 5'-AAATTTCATATGTTACTGAGCCGTATTTAG-3' corresponding to the translation initiation and termination codons (underlined), respectively. PCR was carried out in a 50 μL volume containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 100 ng of template DNA, 100 pmole of each primer, and 2.5 U of Taq DNA polymerase. DNA was amplified as follows: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C, and a final extension at 72°C for 5 min. The PCR product was digested with *Nde*I and cloned into the pRSET-B vector to generate the TmPecN-expressing plasmid, pRBTmPecN.

Denaturation procedure for purification of TmPecN. pRB-TmPecN was transformed into *E. coli* BL21. TmPecN expression was induced by adding 0.7 mM IPTG at 37°C for 4 h. The cell pellet was resuspended in 4 mL of lysis buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole], and sonicated for 4 min on ice. The crude extract containing the TmPecN was heat treated at 70°C for 20 min, and insoluble debris was removed by centrifugation at 10,000×*g* for 10 min. The soluble fraction was removed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Kim et al., 2000).

Pectinase activity assay. Pectinase activity was assayed in a 300 μL reaction mixture containing 0.5% citrus pectin, 50 mM sodium phosphate buffer (pH 6.5), 0.6 mM $CaCl_2$ and 2 μL of partially purified TmPecN. Following incubation at 85°C for 30 min, the amount of reducing sugars released during the reaction was determined by dinitrosalicylic acid (DNS) method (Kim, 2014).

Enzymatic properties of TmPecN. Optimum pH for pectinase activity was determined in a series of 100 mM McIlvaine buffers with pH values ranging from 4.0 to 6.0, and sodium phosphate buffer with pH from 6.0 to 8.0. Optimum temperature for pectinase activity was determined by incubating the reaction tubes in a series of water baths at temperatures ranging from 80 to 100°C.

To test thermostability, TmPecN was incubated at 80, 85, 90, 95, and 100°C. The enzyme was incubated for 180 min, extracting at 30 min intervals, and the residual enzyme activity was determined. To determine the effects of various metal ions on enzyme activity, TmPecN was incubated with 1 mM Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, or Zn^{2+}. After treatmentat different temperatures and with metal ions, enzyme activities were assayed as described above.

Results and Discussion

Production of intact form of TmPecN. Previously, the *TmPec* gene was cloned into a pRSET expression vector to fuse with a histidine tag. The biochemical properties of this purified recombinant protein were characterized (Kim, 2014). In the present study, the *TmPec* gene was cloned into an *Nde*I site of the pRSET vector to be expressed in its intact form. The overexpressed TmPecN was partially purified by heat-denaturation, and the biochemical properties of the protein were analyzed.

It was previously reported that ORF of *TmPec* gene is 1,104 bp long and encodes 367 amino acid residues with a molecular weight of 40,605 Da (Kluskens et al., 2003). The first 27 amino acids of TmPec were predicted to be the signal sequence. In the present study, the putative signal sequence of 27 amino acids was excluded to obtain a mature intact form of TmPec protein (1,026 bp), by using translation initiation and termination-specific primer sets for the PCR amplification. The PCR product was digested with *Nde*I and introduced into the pRSET-B vector to generate pRBTmPecN.

The pRBTmPecN was transformed into *E. coli* BL21, and TmPecN expression was induced and partially purified by heat-denaturation. The estimated molecular weight of TmPecN was approximately 36 kDa (Fig. 1), which is in accordance with the value calculated based on the amino acid sequence.

Pectinase activity of TmPecN was assayed in a reaction mixture containing 0.25% citrus pectin. Reducing sugars were released

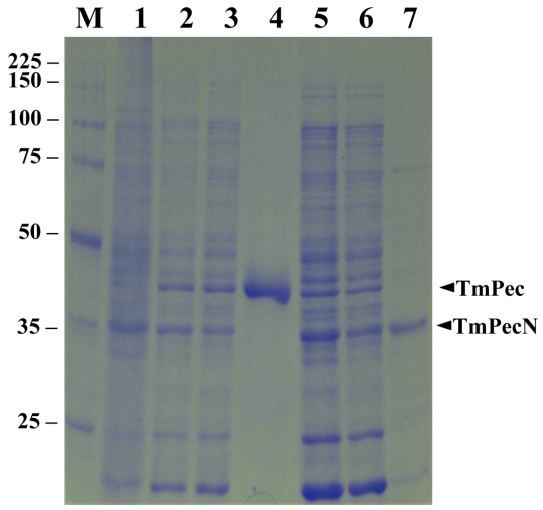

Fig. 1 Expression of TmPecN and recombinant TmPec in *E. coli.* The crude extract containing the TmPecN was partially purified by heat denaturation (70°C, 20 min). After centrifugation at 10,000×*g* for 10 min, the soluble fraction was removed and analyzed by 10% SDS-PAGE.
Lane M: Molecular weight marker.
Lane 1: Total extract from *E. coli* harboring pRSET plasmid only.
Lane 2: Total extract from *E. coli* harboring pRBTmPec.
Lane 3: Soluble fraction from *E. coli* extract harboring pRBTmPec.
Lane 4: Purified recombinant TmPec.
Lane 5: Total extract from *E. coli* harboring pRBTmPecN.
Lane 6: Soluble fraction from *E. coli* extract harboring pRBTmPecN.
Lane 7: Partially purified TmPecN.

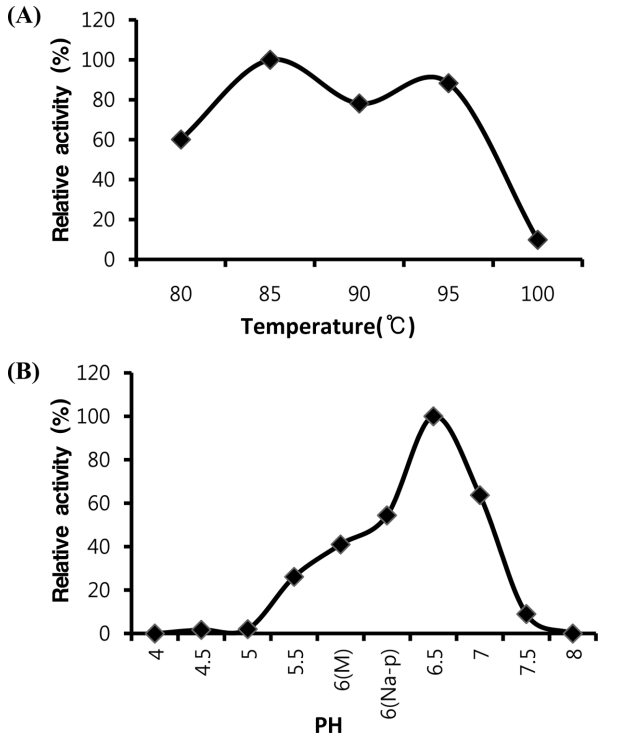

Fig. 2 Optimal temperature and pH for TmPecN activity. (A) Optimal temperature. Citrus pectin (0.5%) was incubated for 30 min with 2 μL of TmPecN at various temperatures in a 300 μL reaction mixture containing 50 mM sodium phosphate buffer (pH 6.5) and 0.6 mM $CaCl_2$. (B) Optimal pH. For the pH test, 50 mM McIlvaine buffer (pH 4-6), sodium phosphate buffer (pH 6–8) were used. Citrus pectin (0.5%) was incubated at 85°C for 30 min with 0.2 μL of TmPecN at various pH values in a final volume of 300 μL. The data represent the mean of three independent experiments.

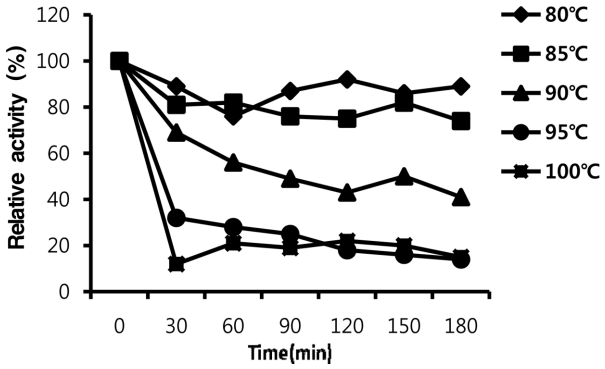

Fig. 3 Thermostability of TmPecN. The TmPecN was incubated at 80, 85, 90, 95, and 100°C. The enzyme was extracted every 30 min for 180 min and its residual activity was determined. The data represent the mean of three independent experiments.

Table 1 Effect of metal ions on TmPecN activity

Metal ions	Activity (%)
control	100.0
Ba^{2+}	43.2
Ca^{2+}	128.4
Cd^{2+}	78.0
Co^{2+}	78.4
Cu^{2+}	119.0
Fe^{3+}	70.9
K^{+}	90.2
Mg^{2+}	78.4
Mn^{2+}	66.2
Na^{+}	94.0
Zn^{2+}	68.8

from citrus pectin, showing that TmPecN possesses pectin degradation activity.

Biochemical properties of TmPecN. TmPecN activity was tested at pH 6.5 between 80 and 100°C to determine its optimum temperature. TmPecN had the highest activity at approximately 85°C (Fig. 2A), and retained more than 80% of its activity up to 95°C. Optimal pH for TmPecN was found to be at 85°C in the pH range between 4.0 and 8.0. TmPecN showed the highest activity at around pH 6.5 (Fig. 2B) and retained more than 60% of its activity in the pH range of 6.0–7.0.

Thermostability of TmPecN. To determine thermostability, TmPecN was incubated at 80–100°C. The enzyme was incubated for 180 min, and its residual activity was determined at 30 min intervals. TmPecN retained over 80% of its activity after 180 min at 85°C; however, the activity decreased after 30 min at 95°C (Fig. 3).

Effect of metal ions on the activity of TmPecN. To determine the effect of metal ions on the activity of TmPecN, enzyme assay was carried out in the presence of various metal ions at 1 mM concentration. The activity of TmPecN was increased to 128.4% by Ca^{2+}, but significantly inhibited by Ba^{2+} and Mn^{2+} (Table 1). Effect of Ca^{2+} was assayed at different $CaCl_2$ concentrations. TmPecN showed the highest activity at 0.6 mM $CaCl_2$, but further increase in $CaCl_2$ concentration resulted in reduction of pectinase activity (data not shown).

Previously, *TmPec* gene was cloned into *EcoR*I site of pRSET expression vector to fuse with a histidine tag (Kim, 2014). The recombinant TmPec contained extra 44 amino acids at the N-terminal region, including a 6-His region, T7 gene 10 leader, and a polylinker site derived from the pRSET-B vector. The extra 44 amino acids might affect the biochemical properties of the recombinant TmPec. The recombinant TmPec showed highest activity at around 85°C, and at around pH 6.5, which was slightly different from the previous study reporting optimal activity at 90°C and pH 9.0 (Kluskens et al., 2003).

In the present study, the intact form of TmPecN showed the highest activity between 85 and 95°C, and a pH of approximately 6.5. It was also stable at temperatures below 85°C. Therefore, the biochemical properties of TmPecN are comparable to those of the recombinant protein described in our previous report (Kim, 2014). These results suggest that the purification by heat-denaturation procedure provides a simplified method for thermostable pectinase preparation. The thermostable pectinase described in this study has excellent potential for industrial-scale biomass degradation.

Further studies addressing the practical applications of thermostable pectinases are in progress.

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