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Isolation of Cysteine Protease Actinidin Gene from Chinese Wild Kiwifruit and its Expression in *Escherichia coli*

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Abstract The actinidin (EC 3.4.22.14) found in kiwifruit is a cysteine protease. In order to obtain the actinidin gene from the Chinese wild kiwifruit, primers were designed on the basis of the actinidin gene of *Actinidia deliciosa*, the New Zealand kiwifruit. The 1.2 kb DNA fragment was acquired from the total RNAs of Chinese wild kiwifruit via reverse transcription polymerase chain reaction (RT-PCR), and its DNA sequence was analyzed. Its sequence was determined to share 98.4% homology with the actinidin gene of *A. deliciosa*. In order to verify the actinidin gene isolated from the Chinese wild kiwifruit in *Escherichia coli*, the mature gene was amplified via PCR and expressed in *E. coli* under the control of the T7*lac* promoter. The actinidin was expressed in *E. coli* as inclusion bodies, which were solubilized with urea and refolded. The protease activity of the refolded protein was approximately twice as high as that of *E. coli* BL21 (DE3).

Keywords: Chinese wild kiwifruit, actinidin gene, protease activity, *E. coli* BL21 (DE3)

Introduction

The kiwifruit, also referred to as the Chinese gooseberry, originates from the Yangtze River Valley of northern China and Zhejiang Province, on the coast of eastern China. As kiwifruit was reported to harbor a strong protease (1), research has been conducted into its molecular biological properties (2-5) and its purification from pulp (6-9).

Actinidin, a cysteine protease contained in kiwifruit, was detected at a high concentration (5, 10), and this increased during ripening and softened the fruit (11). It harbors an N-terminal peptide consisting of 126 amino acids, a mature peptide of 220 amino acids, and a C-terminal peptide comprised of 33-34 amino acids (4, 12). The structure of actinidin is similar to that of papain, but the enzyme activities are different (13). The proteolytic activities of kiwi protease are dependent upon substrates, pHs, and temperatures (1, 10, 14, 15). Generally, optimal proteolytic activity to casein is observed in a pH range of 7.0 to 7.6 and a temperature range of 40 to 62°C (6, 7, 16, 17). Its proteolytic activity is inhibited by $HgCl_2$, $MnSO_4$, cobalt ion, phenylmecrcuric acetate, and leupeptin (6, 7, 12, 17), but is increased by cysteine, EDTA, and the sodium and iron II ions (6, 7, 17).

Actinidin belongs to the same category of cysteine proteases as papain, chymopapain, bromelain, ficin, aleurain, and caricain (5, 6). Proteases in the papain family have many industrial applications; actinidin can potentially serve as a substitute for the papain family, as a meat tenderizer, an agent to extract protein from bones, and also in leather processing (6, 16). For these reasons, heterologous gene expression of the actinidin gene was attempted in tobacco and yeast (12, 18, 19), but the expression levels were low. The phenotypes and growth patterns of recombinant tobacco and yeast were altered. The successful production of recombinant actinidin in microorganisms has yet to be reported. In this study, the primitive actinidin gene was isolated and identified from the Chinese wild kiwifruit and was expressed in *Escherichia coli*.

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Materials and Methods

Chinese wild kiwifruitz Wild kiwifruits were acquired from the Yellow Mountain (Hwang Mt.) region of China, and New Zealand kiwifruit, Hayward, was obtained from a grocery store in Anseong, Korea on Aug. 2000.

Bacterial strains, vectors, enzymes, and growth conditions *E. coli* DH5α [*supE44*, *ΔlacU169* (80 *lacZΔM15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*] and pGEM-T Easy vector (Promega, Madison, WI, USA) were used for cloning. *E. coli* BL21 (DE3) [F-, *ompT*, *hsdSB* (rB-mB-)], *gal* [*c I857*, *ind1*, *Sam7*, *nin5*, *lacUV5-T7gene1*), *dcm* (DE3)], and pET-25b (+) vector were used to express the recombinant gene. All *E. coli* strains were grown in LB medium at 37°C and ampicillin (50 μg/mL) (Sigma, St. Louis, MO, USA) was added for the selection of recombinant strains. Restriction enzymes were purchased from MBI Fermentas (Hanover, MD, USA) and used as recommended by the manufacturer.

Amplification of the actinidin gene via reverse transcription polymerase chain reaction (RT-PCR) In order to isolate the actinidin gene from Chinese wild kiwifruit, total RNAs were extracted (20) using guanidinium thiocyanate (Sigma). These total RNAs were employed for cDNA synthesis using a first-strand cDNA synthesis kit (MBI). The synthesized cDNAs were amplified with oligonucleotide primers, which were designed on the basis of the published sequences of the actinidin gene (4, 21) and synthesized at Bioneer (Daejeon, Korea). The sequences of the forward and reverse primers were 5'-GAGAACAAAAATGGGTTTGC- 3' (the start codons underlined) and 5'-TTCCTAAGCGCTGTACCTCT-3' (the

stop codon is underlined), respectively. PCR was conducted using a Minicycler™ (PTC-150; MJ Research, Watertown, MA, USA) and carried out over 30 cycles (initial denaturation at 94°C for 3 min, denaturation at 94 °C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min) with a final 10-min extension step at 72°C.

Nucleotide sequence analysis The nucleotide sequence was determined by an ABI PRISM 377 DNA Auto Sequencer with an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kits (Appied Biosystems, Foster, CA, USA). The sequences were analyzed using a Bio Max STS 45i (Shelton-IBI, Peosta, IA, USA) with a Reader™ DNA sequencing kit (MBI). The analyzed nucleotide sequences were compared with those in the databases, via a BLAST search.

Amplification of the actinidin mature gene and its expression in *E. coli* In order to amplify the mature actinidin gene of Chinese wild kiwifruit, the primers were synthesized at TaKaRa-Korea Bio Medicals (Seongnam, Korea) on the basis of the mature gene sequence of *A. deliciosa*. The primers were as follows: 5'-AAACCATGG TATTGCCGAGTTA-3' (the *Nco*I site is underlined) and 5'-GGGTGATTCTAGTTGTTGTACT-3' (the stop site is underlined). PCR was conducted via the conditions described above. The amplified mature fragment was subcloned into pGEM-T Easy vector and then inserted into the pET-25 (+) vector and transformed into *E. coli* BL21 (DE3) in accordance with the standard protocols (20). For the expression of actinidin in *E. coli*, 1 mL of a cultured transformant harboring the actinidin gene was transferred into 50 μL LB medium containing ampicillin (50 μg/mL) and incubated at 37°C with shaking. When the cell density reached a level of $0.3/A_{600}$, isopropyl-β-D-1-thiogalacto-pyranoside (IPTG; Sigma) was added to the medium at a final concentration of 1 mM and the cells were harvested after 4 hr of induction and analyzed via sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE; Sigma).

Protein assay Using bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA, USA) as a standard, the protein concentrations were determined via the Bio-Rad Bradford method.

Solubilization of inclusion bodies The solubilization of inclusion bodies was conducted using a modified version of Margetts's method (22). The expressed protein was denatured with 8 M urea containing 0.01 M dithiothreitol (DTT; Sigma) and 50 mM Tris-HCl (Sigma), pH 7.8, and refolded by dialysis in refolding buffer [100 mM Tris-HCl, pH 7.8, 5 mM $CaCl_2$ (Sigma), 5 mM DTT, 5 mM Cysteine (Sigma), 150 mM NaCl (Sigma)].

Specific protease activity assay Specific protease activity was determined via a modified version of Yamaguchi's method (16, 17). One tenth %(w/v) casein in 0.1 M sodium phosphate (Sigma) buffer (pH 7.0) was used as the substrate. One mL of substrate was incubated with 0.2 mL of enzyme solution containing 5 mM cysteine and 5 mM ethylenediaminetetraacetic acid (EDTA; Sigma) for 20 min at 40°C. The reaction was halted via the addition of 2 mL of 5% trichloroacetic acid (TCA; Sigma) and the precipitated protein was removed with Whatman filter paper (No. 2). The absorbance of the filtrates was measured at A_{280} using a Beckman DU® 650 spectrophotometer (Fullerton, CA, USA). One unit of enzyme activity was defined as the quantity of enzyme inducing a change of 0.001 A_{280} per min under the above conditions.

Nucleotide sequence accession number The DNA sequence of the actinidin gene of Chinese wild kiwifruit was deposited into the GenBank database under the accession number AF343446.

Results and Discussion

Physical properties of Chinese wild kiwifruit The Chinese wild kiwifruit was compared with the New Zealand kiwifruit with regard to its physical properties, including size, weight, pH, and sweetness. Three samples were selected randomly from Chinese wild kiwifruits and New Zealand kiwifruits, respectively, and the data were averaged (Table 1, Fig. 1). The size and weight of the Chinese wild kiwifruit were 5 and 8 times less than those of the New Zealand kiwifruit, respectively. The values of pH and sweetness were, however, approximately identical.

Cloning of the PCR product and nucleotide sequence analysis The amplified 1.2 kb fragments from the synthesized cDNA were subcloned into pGEM-T Easy vector and transformed into *E. coli* DH5α. The vector was designated pWACT-1, and was employed for nucleotide sequence analysis. The DNA sequences were analyzed by a Blast search of the National Center for Biotechnology Information (NCBI) database and the result shows that the cloned gene was 98.4% identical with the actinidin gene of *A. deliciosa*. The translated amino acid sequences obtained by the NCBI ORF Finder showed 98.7% homology with those of actinidin from *A. deliciosa*. For the mature peptide region, the amino acid sequences evidenced 98.6% homology. As a result of nucleotide sequence analysis (Fig. 2), the base sequences at A^{51}, A^{312}, A^{313}, G^{367}, G^{518}, A^{533}, A^{623}, T^{629}, G^{675}, C^{800}, A^{806}, G^{815}, C^{836}, G^{855}, T^{866}, T^{884}, and A^{1088} of the amplified 1.2 kb fragment from Chinese wild kiwifruit were not concordant with the actinidin DNA sequence of *A. deliciosa*. Especially, the A^{313}, G^{367}, G^{675}, G^{815}, and G^{855} caused different amino acids for the amplified 1.2 kb fragment from Chinese wild kiwifruit (Arg^{105}, Val^{123}, Val^{226}, Gin^{272}, and Val^{286}) and the actinidin gene of *A. deliciosa* (Gly^{105}, Phe^{123}, Leu^{226}, His^{272}, and Ile^{286}).

Table 1. Physical properties of Chinese wild kiwifruit and New Zealand kiwifruit, Hayward

Characteristics	Chinese wild kiwifruit	New Zealand kiwifruit
Size (cm)	2.73 (L) × 2.37 (W)	6.43 (L) × 4.87 (W)
Weight (g)	11.3±1.30	99.2±0.32
pH	2.79±0.01	3.07±0.01
°Bx	15.4±0.09	15.7±0.09

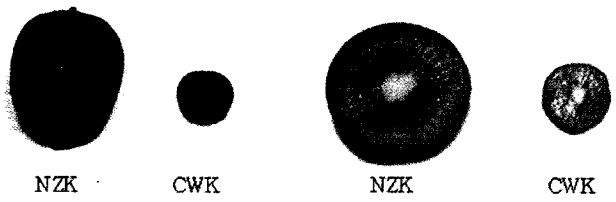

Fig. 1. Chinese wild kiwifruit and New Zealand kiwifruit, Hayward. CWK, Chinese wild kiwifruit; NZK, New Zealand kiwifruit.

Construction of the actinidin mature gene and its expression in *E. coli* In order to express the actinidin gene in *E. coli*, the mature gene was PCR amplified from pWACT-1. The amplified 600 bp fragments inserted into the pET-25b (+) vector, designated as pWMACT-1, was confirmed via digestion with *Nco*I and *Bam*HI and the DNA sequencing of the N-terminal fragment (data not shown). The vector was called pET-WM1 (Fig. 3). IPTG was added to a culture of *E. coli* BL21 (DE3) harboring pET-WM1, and the proteins were analyzed via 15% SDS-PAGE (Fig. 4). The overproduced protein was detected in the cell pellets. This result showed that actinidin was generated as inclusion bodies in *E. coli*, similarly to the

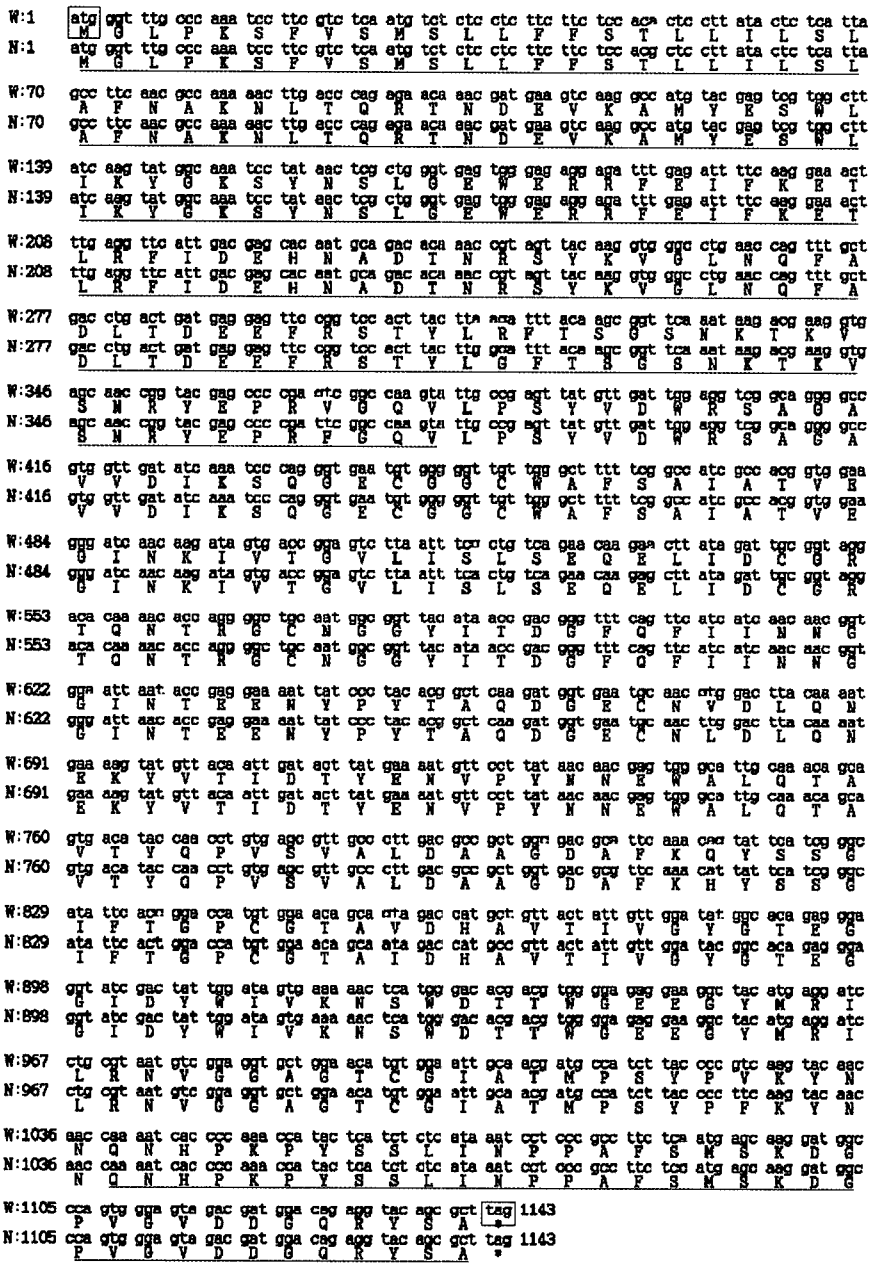

Fig. 2. Comparison of DNA sequences and amino acid residues between the amplified 1.2 kb fragment from Chinese wild kiwifruit and actinidin gene of *A. deliciosa*. W, Chinese wild kiwifruit; N, *A. deliciosa*; Block, mismatch parts of DNA sequences and amino acids; Squares, start and stop codons of actinidin gene of *A. deliciosa*; Underline, N- and C-terminal extension of 126 and 34 amino acids of actinidin gene of *A. deliciosa*.

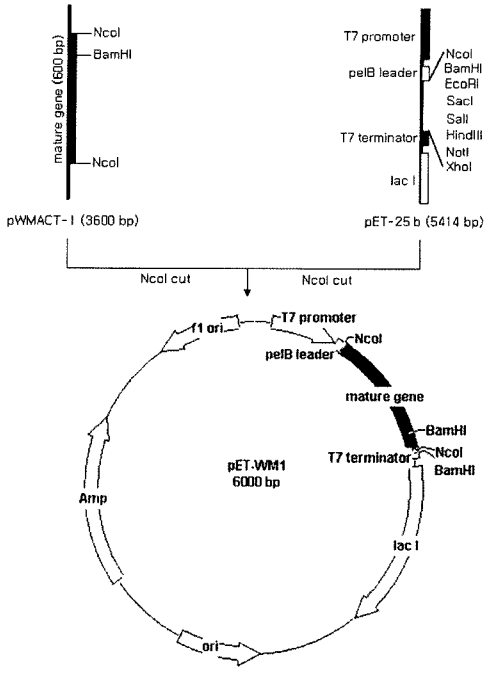

Fig. 3. Construction of the actinidin expression vector, pET-WM1.

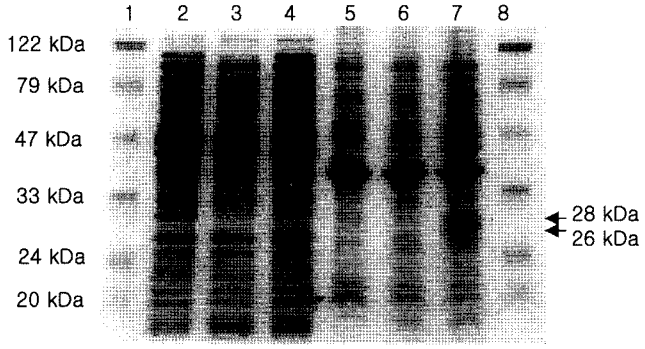

Fig. 4. Analysis of the expressed protein by SDS-PAGE. Lane 1, 8 standard protein marker; lane 2, cell extract of BL21 (DE3); lane 3, cell extract of BL21 (DE3)/pET-25b; lane 4, cell extract of BL21 (DE3)/pET-WM1; lane 5, cell pellets of BL21 (DE3); lane 6, cell pellets of BL21 (DE3)/pET-25b; lane 7, cell pellets of BL21 (DE3) /pET-WM1.

previously reported papain (23). The sizes of the proteins on SDS-PAGE were 26 and 28 kDa. It was assumed that the full and truncated gene products of actinidin were generated in *E. coli.*

Protease activity of the recombinant actinidin produced in *E. coli* The protease activity of the cell extract of *E. coli* BL21 (DE3)/pET-WM1 was lower than that of *E. coli* BL21 (DE3) (data not shown). It was suspected that the formation of inclusion bodies in *E. coli* caused this lower activity. In order to restore the protease activity of actinidin forming inclusion bodies in *E. coli*, the proteins were denatured with 8 M urea and then renatured via dialysis with a lowering of the urea concentration. The

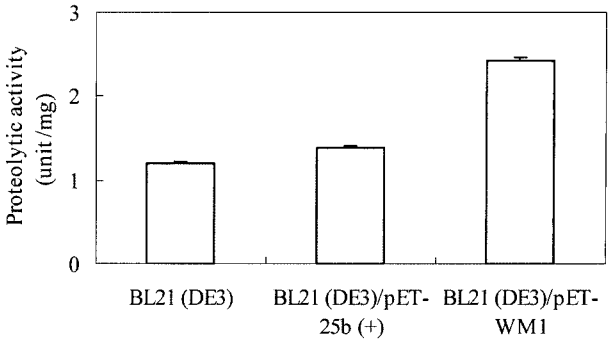

Fig. 5. Specific protease activity of the expressed actinidin in *E. coli.*

protease activity of the refolded protein was approximately twice that of the cell extract of *E. coli* BL21 (DE3) (Fig. 5). By way of comparison, the reported protease activity of kiwifruit extract (Hayward) on a casein substrate has been determined to be 12.0 U/mg (16), and although its activity was recovered by refolding buffer, the value was relatively low. The reasons for this are believed to be attributable to protein degradation during the reactivation process, incorrect protein refolding or the lack of posttranslational modifications, such as glycosylation, in *E. coli.*

In conclusion, the primitive actinidin gene, which evidences a high degree of homology with the actinidin gene of *A. deliciosa,* was obtained and examined herein. Despite geographic differences and breed improvements, we confirmed that the actinidin gene of *A. deliciosa* and Chinese wild kiwifruit are almost identical. Although actinidin was synthesized as inclusion bodies in *E. coli*, the data indicated that the refolded actinidin evidenced protease activity, and that the overproduction of actinidin in microorganisms was possible.

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