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Properties of recombinant novel cinnamoyl esterase from *Lactobacillus acidophilus* F46 isolated from human intestinal bacterium

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ABSTRACT

A novel cinnamoyl esterase (CE) gene (741 bp) from *Lactobacillus acidophilus* F46 was cloned and expressed with a His_6-tagged protein in *Escherichia coli*. The recombinant CE consists of 247 amino acids and shows the highest similarity of 70% with CE sequences of *Lactobacillus jonnosonii*. The purified enzyme presents a single band on SDS- PAGE with an apparent molecular mass of about 27 kDa. It showed the highest activity at pH 7.5 and 50 °C. Also, it presented stability over a range of pH 4.0–9.0, and thermal stability of the enzyme decreased rapidly at temperatures above 50 °C. At a concentration of 5 mM liter^{-1}, $CaCl_2$, $CuSO_4$, $FeSO_4$, and $MnSO_4$ reduced the activity by 75.9, 55.4, 59.8, and 73.8% respectively, which indicates different inactivation resistance compared to other CE from lactic acid bacteria. Under standard conditions (pH 7.0 and 37 °C) with chlorogenic acid as a substrate, the enzyme exhibited a K_m of 4.89 mmol liter^{-1} and V_{max} of 1250 μmol/min^{-1} mg^{-1} of protein. The purified recombinant CE showed a very strong conversion activity of chlorogenic acid to caffeic acid, and 95.3% of the chlorogenic acid was consumed. At the same time, the caffeic acid reached a concentration of 8 mM at 60 min whereas the chlorogenic acid remained the same at only 0.8 mM. This high conversion ratio of the CE from lactic acid bacteria strongly indicates that this lactic acid bacteria with strong CE enzymatic activity has the potential for industrial applications.

1. Introduction

Although phenolic compounds exist abundantly in various foods, they have been considered nutritionally undesirable components for a long time due to their instability in foods and inhibitory properties such as precipitation, inhibition of digestive enzyme, and reduction of vitamins and minerals affecting food quality [1–3].

However, due to the great interest of phenolic compounds in foods because of their antioxidant activity causing important health functions in the prevention or pretreatment on various diseases related with carcinogenesis and mutagenesis, many studies have been done to utilize them efficiently [4,5].

Moreover, some phenolic compounds such as chlorogenic, caffeic, ferulic, sinapic, and p-coumaric acids called hydroxycinnamic acids are found in fruits and vegetables and represent a major class of phenolic compounds that showed remarkably high levels of antioxidant activity in vitro [6,7] with additional preventive effects on atherosclerosis, cardiovascular disease, and cancer [8]. Additionally, caffeic acid also recently showed strong insulin secretion stimulation activity from pancreatic β-cells and led to suppressed blood glucose levels in vivo in diabetic mice, thus indicating possible safer antidiabetic agents [9–11]. However, those phoenolic compounds often face great problems in the human intestines due to their non-digestive properties.

Cinnamoyl esterases (CE) are carboxyl ester hydrolases (EC 3.1.1.1) that degrade hydroxycinnamate esters and sugars present in the plant cell walls, producing very small final products of phenolic acids such as caffeic acid and ferulic acid [12,13]. CE plays an important role in releasing free hydroxycinnamic acid and derivatives from fibers present in food at the intestines of mammalian digestive system and showed high level antioxidant activities contributing to human health. Although CE activity was derived from the mucosa in the small intestines, a higher level of activity was detected in samples obtained from the human feces cell-free extracts [5]. This result suggests that the CE activity produced by intestinal microorganisms is an important factor in releasing esterified phenolic compounds (free hydroxycinnamic acid), which have been shown to have positive effects on human health. [12,14]. CE activity is well known in a wide range of bacteria and fungi including genera found in the digestive tracts of animals and humans [15–17]. *Lactobacillus johnsonii* LJ1228, which displays strong ferulic acid esterase activity in stool samples, has been isolated from

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diabetes-resistant biobreeding rats, thereby demonstrating that CE activity toward ethyl ferulate and chlorogenic acid might be very strongly associated with diabetic resistance [18].

In our previous study, we were able to successfully isolate four strains from human feces. Among them, we found that the highest conversion ratio from chlorogenic and to caffeic acid was observed in the *L. acidophilus* F46 strain. Even though *L. acidophilus* is one of the most widely recognized probiotics that is extensively used in the food and pharmaceutical industries [19,20], we could not find any CE-related information from this strain to the best of our knowledge. Only a single previous report showed that a feruloyl esterase from the L. *acidophilus* was found and indicated that the feruloylated sugar esters can be used as the phenol compounds [21]. However, it is very confusing as to whether the obtained feruloyl esterase showed CE activity responsible for our obtained *L. acidophilus* F46 due to the lack of explanation about the properties on CE activity in hydroxycinnamate substrates. Moreover, the N-terminal sequence analysis of feruloyl esterase from *L. acidophilus* showed a very high homology on L-LDH of 95% rather than CE or FAE. Even when the genome sequence of *L. acidophilus* NCFM, which published recently and was searched for CE, no matched CE protein was found.

In this study, a CE gene was cloned from human origin *L. acidophilus* F46, and we examined its sequences by comparing other CE or FAE genes. Additionally, the properties of the novel CE that showed high-level CE activity from L. acidophilus F46 were evaluated after cloning, expression, and purification.

2. Materials and methods

2.1. Bacterial strains, media, plasmids and chemicals

L. acidophilus F46 isolated with a high cinnamoyl esterase activity from intestine bacteria was incubated in MRS media (Difco, Detroit, MI, USA) for 72 h at 37 °C and kept in our lab at 80 °C. *Escherichia coli* strain JM109 was used as the host for gene cloning and plasmid propagation, and *E. coli* BL21 (DE3) for protein expression with pET-21a and pET-28 was used as an expression vector. Chlorogenic acid, caffeic acid, ethyl ferulate, and IPTG (isopropyl-L-thio-β-D-galactopyranoside) were purchased from Sigma Chemical (St. Louis, MO, USA). The restriction enzyme, NdeI and XhoI were purchased from Promega and BamHI, EcoRI was purchased from from Roche (Mannheim, Germany). Methyl ferulate (MFA), methyl caffeate (MCA), and methyl p-coumarate (MpCA) were purchased from Apin Chemicals Ltd (Oxon, UK); methyl sinnapate (MSA) was obtained from Santa Cruz Biotechnology Inc.

2.2. DNA manipulation and nucleotide sequencing analysis

The nucleotide sequences were analyzed via a dye terminator cycle-sequencing reaction by following the supplier's instructions using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI Prism 377 sequencer (Applied Biosystems). The resulting sequence data were then analyzed using the DNASIS and PROSIS programs (HITACHI Software Engineering Co., Japan), respectively. A homology search was conducted using the World Wide Web server for BLAST searching maintained at the National Center for Biotechnology Information (Altschul, S. F.). A multiple sequence alignment of related amino acid sequences was performed using the CLUSTAL V program (Higgins, D. G.).

2.3. Cloning and expression of putative CE genes by PCR

Four oligonucleotide primers with each restriction sites of BamHI, EcoRI, NdeI, or XhoI were designed based on the genomic sequence of L. acidophilus NCFM encoding alpha/beta hydrolase gene (GenBank accession number YP_194675) as shown in Table 1. The genomic DNA of *L. acidophilus* F46 was isolated by using the genomic DNA extraction kit (Intron, Korea), and then, we used it as a template for the polymerase chain reaction (PCR). PCR was performed with a Taq DNA Polymerase PCR kit (GeneAll, Korea) for 27 cycles of: 95 °C for 60 s, 54 °C for 30 s, and 72 °C for 1 min. Four PCR amplified fragments with approximately 700 bp (A, CEacidoN and CEacidoX; B, CEacidoB and CEacidoX; C, CEacidoB and CEacidoE; and D, CEacidoN and CEacidoE) were obtained and directly inserted into the pET21a or the pET28a expression vector, respectively, named A, pET28a-CEacido 1; B, pET28a-CEacido 2; C, pET21a-CEacido 3; and D, pET21a-CEacido 4. Next, they were transformed into *E. coli* BL21(DE3) competent cells by using a heat shock procedure. The obtained recombinant *E. coli* (DE3) under the control of a strong T7 promoter was grown in LB broth supplemented with 100 μg/mL ampicillin or kanamycin for pET21a or pET28a, respectively. After addition of 1 mM IPTG when OD_{600} reached 0.8 (approximately 3 h), then cultivation was continued until 18 h at 37 °C with reciprocal shaking. After induction, the cells were harvested by centrifugation (3000 × g, 15 min, 4 °C), washed twice with 0.85% NaCl solution, and suspended in Tris-HCl buffer (10 mM, pH 7.0). After disruption by gentle sonication (7 cycles, 20 s) with 50 mM PBS on ice, the cell debris was removed from the supernatant by centrifugation (10,000 × g, 20 min, 4 °C) to obtain the crude extract. The expression of the recombinant CE in *E. coli* was confirmed by SDS-PAGE on 12% polyacrylamide after staining with Coomassie Brilliant Blue R-250.

Table 1
Oligonucleotide primers used in this study.

Primer	Nucleotide sequence
CEBamN	AAAAAGGATCCATGTCTCGCATTACAA TTGAG
CEEcoRC	AAAAAGAATTCTTAAAACGTTGCAGGTTTTAA AAATTGC
CENdeN	AAAACTCGAGAAATAGGGGCTTCAAAAATTC
CEXhoC	AAAACTCGAGAAATAGGGGCTTCAAAAATTC

The restriction enzyme site is underlined.

2.4. Purification of recombinant CE by his_6-tag sequences

The crude extract containing the expressed recombinant CE was prepared as described above and purified by one-step chromatography. The prepared crude extract was applied into the Ni-sepharose (TM HP) resin previously equilibrated with buffer A (50 mM PBS, 300 mM NaCl, 2 mM imidazole, pH 7.0) according to manufacturer's recommendations, and the recombinant CE was eluted with 150 mM imidazole. After dialysis twice against 50 mM PBS buffer containing 300 mM NaCl (pH 7.0), the obtained protein solution was used as the purified CE enzyme. The purity and apparent molecular mass were determined by SDS-PAGE, and the protein concentration was determined using a Bio-Rad protein assay kit with BSA as standard.

2.5. Recombinant CE activity

Recombinant CE activity was conducted at 37 °C for 10 min in a solution of 50 mM phosphate buffer (pH 7.5) containing chlorogenic acid (10 mM) as substrate with 1 μg of purified enzyme. The reaction was terminated by heating for 3 min in a boiling water bath. The reaction mixture was then analyzed by HPLC (High Performance Liquid Chromatography) and quantified by comparison with chlorogenic acid standards. HPLC analysis was performed on a Waters system 1525 binary HPLC pump (Bedford, MA, USA) with evaporative light scattering detector (Alltech ELSD 800, Nicholasville, KY, USA) using a Inersil ODS-3 V column (150 × 4.0 mm i.d., 5 μm). The mobile phase was 0.5% formic acid