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Characterization of a Carboxylesterase from *Burkholderia ferrariae* L-2 which Enhances the Flavor of Cheese

Tamio MASE*, Noriko HISAMATSU*, Yuu MURATA* and Etsuko HIROSE*

Abstract

A carboxylesterase (CE) derived from *Burkholderia ferrariae* isolated from the digestive juice of *Nepenthes alata*, was purified from the culture filtrate, and CM-Toyopearl 650M, DEAE-Toyorearl 650M, Butyl-Toyopearl 650M, and Toyopearl HW-55F chromatography. The purified enzyme appeared as a single band with a molecular mass of 92 kDa by SDS-PAGE. The optimum temperature and pH were approximately 50℃ and 7.0, respectively. According to cheese flavor formation by milk fat hydrolysis, the esterase was suggested the possibility as a food additive in dairy food processing.

Introduction

Carboxylesterase (EC 3. 1. 1. 1) are enzymes that catalyze the hydrolysis of carboxylic ester such as methylbutyrate and are widely found in animals, plants, and microorganisms. CE can also hydrolyze short chain triglycerides and release short chain fatty acids which have important roles in the natural aroma of foods (Langrand *et al*., 1988). As microorganisms produce CE or lipase (EC 3. 1. 1. 3) with diverse enzymatic properties and substrate specificities, they represent a valuable source of enzymes with potential application in the food industry (Tanaka *et al*., 1993). In the dairy industry, CE or lipase are used for flavor development in specific cheese types, flavor enhancement, acceleration of the cheese ripening process, and for the production of cheese-like products (Arnold *et al*., 1975). In particular, pregastric esterase (Kwak *et al*., 1989) is an important enzyme for the production of enzyme modified cheese which is used to formulate foods which require a cheese note. The short-chain fatty acids released by pregastric esterase not only function as flavor components themselves, but also serve as

* 生活科学部　管理栄養学科

precursors for other flavor components. We were therefore interested in identifying a microbial CE or lipase with qualities similar to pregastric esterase, and screened 40 bacteria for CE production. From this screening, *Burkholderia ferrariae* L-2 was identified as an efficient CE producer. In this study, we purified and characterized the extracellular CE produced by the strain L-2, and examined the possibility as the dairy industry use.

Materials and Methods

Microorganisms The bacteria strain L-2 used in this study was newly isolated from the digestive juice in the pitcher of *Nepenthes alata* which was gathered from Higashiyama Botanical Garden in Nagoya, Japan.

Strain L-2 was identified by gene sequence analysis based on 16S rDNA. After genomic DNA was extracted InstaGene Matrix (BiO Rad, CA, USA), the 16S rDNA gene was amplified by PCR using MicroSeq 500 16S rDNA Bacterial Identification PCR Kit (Applied Biosystems, CA, USA). The PCR products were sequenced using ABI PRISM 3100 Genetic Analyzer System (Applied Biosystems, CA, USA). Assembly of the sequence fragments was performed using ChromasPro 1.34 (Technelysium Pty Ltd., Tewantin, AUS). To analyze the sequences, homology analysis was performed by BLAST (Altschul *et al*., 1997) against 2 databases, MicroSeq bacterial full gene library V. 0001 (Applied Biosystems, CA, USA and NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed from evolutionary distance calculated by the neighbor-joining method (Saitou & Nei, 1987). Stock cultures were maintained on PEARCORE PLATE COUNT agar (EIKEN CHEMICAL CO., LTD., Tokyo) with 1% glucose (pH 7.0).

Enzyme assay Reaction mixture was containing 2 mL of 1 mM *p*-nitrophenyl acetate solution dissolved in 0.1M phosphate buffer (pH 6.0) and 0.2 mL of enzyme solution. After reaction at 50°C for 20 min, 2 mL of ethanol was added to terminate the reaction. One unit of esterase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min under the conditions.

Cultivation A single colony of L-2 from an agar slant was inoculated into 100-mL shaking flasks containing 20 mL of medium. The medium (pH 5.0) consisted of 1% Polypepton S (Dainippon Sumitomo Pharma Co., Ltd., Japan), 0.5% Bact^{T} Yeast Extract (Wako Pure Chemical Industries, Ltd.), 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7aq$, 0.5% glucose, 0,5% glycerol and 1.5% Tween 80 (Kanto Reagents Chemicals & Biologicals Ltd., Tokyo). After incubation at 35°C for 24 h, 40 mL of the culture was inoculated into a 3-L jar fermentor containing 2 L of the medium described above. The cultivation was performed at 35°C for 72 h with agitation at 350 rpm and aeration at 1.5 L per min.

Purification of Esterase All steps in the purification were performed at 4°C unless otherwise noted. After 72 h of cultivation, the cells were removed by centrifugation and a

crude CE solution was obtained by filtration. The filtrate was concentrated by ultrafiltration using AIV-1010 (Asahi Kasei Co.) with nearly 100% recovery of CE activity. The esterase solution was dialyzed against 0.02 M phosphate buffer (pH 7.0) for 16 hr and applied to a CM Toyopearl 650M column (4 × 20cm) equilibrated with the same buffer. The non-adsorbed active fractions were combined. The solution was applied to a DEAE-Toyopearl 650M column (4 × 20cm) equilibrated with same phosphate buffer. The adsorbed enzyme was eluted with a linear gradient of 0-0.5 M NaCl in phosphate buffer. The active fractions were combined. Solid $(NH_4)_2SO_4$ was added into the active fraction up to 20%. The enzyme solution was applied on a Butyl-Toyopearl 650M column (2.2 × 20cm) equilibrated with 0.02 M phosphate buffer with 20% $(NH_4)_2SO_4$. The adsorbed enzyme was eluted with a linear gradient of 20-0% $(NH_4)_2SO_4$ in phosphate buffer. The active fraction eluted was dialyzed against 0.03 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. The active fraction was subjected to gel filtration on a Toyopearl HW-55 column (2.2 × 92cm) using 0.03 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. The active fractions were combined and dialyzed against a large volume of 0.01 M phosphate buffer (pH 7.0). The resulting dialyzate was used for further experiments as the final enzyme preparation.

Protein assay Protein concentration was measured with Bio-Rad protein reagent (Bio-Rad Laboratories, USA) using bovine serum albumin as the standard. The absorbance at 280 nm was for used for monitoring protein in the column effluent.

Determination of molecular weight SDS-PAGE was performed with a Compact PAGE AE-7305 (Atto Co., Ltd.) using C-PAGEL (Atto Co., Ltd.). To determine the molecular mass, EZ Standard AE-1440 (Atto Co., Ltd.) was used as the protein standards.

Application for dairy products Ten gram of Condensed milk (Morinaga Milk Industry Co., Ltd., Tokyo), 10 mL of distilled water and 20 mL of purified enzyme was mixed and reacted at 50℃. A smoll portion of reaction mixture (0.8 mL) was removed every 30 min, and stopped the reaction in boiling water bath. Chloroform (1.6 mL) was added into the reaction mixture for extraction of free fatty acid. After separated the chloroform layer, chloroform was removed under the vacuum conditions. The remained free fatty acid was measured with NEFA C test WAKO (Wako Pure Chemical Industries, Ltd.). The sensory characteristics of the hydrolyzate was evaluated according preference test as cheese-like flavor. The evaluation panel consisted of fifteen women students aged 22 years of Sugiyama Jogakuen University.

Results and Discussion

A CE produced by a newly isolated bacteria from the digestive juice in the pitcher of *N. alata* was purified from culture filtrate and characterized, and its potential application toward the flavoring of dairy products was examined. The bacteria isolate, designated L-2, was identified as a strain of *Burkholderia ferrariae* from comparative 16S rDNA gene

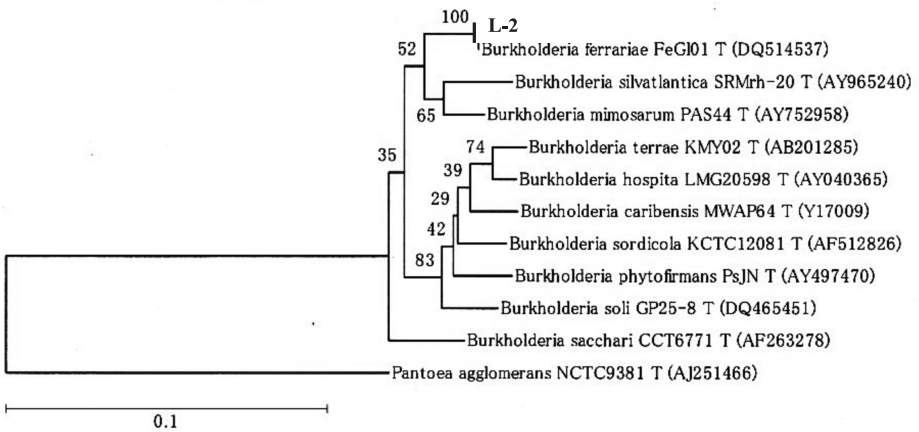


Fig. 1. Neighbor-joining tree derived from 16S rDNA sequences, showing the phylogenetic positions of *Burkholderia ferrariae* and the most closely related *Burkholderia* species.

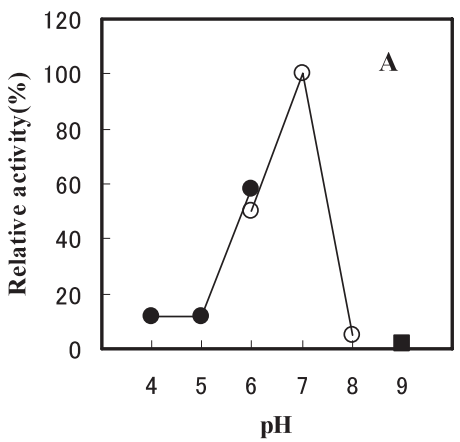
sequence analysis (Fig. 1). *B. ferrariae* isolated from an iron ore in Brazil was reported (Valverdo, *et al.*, 2006) and is known as non-pathogenic bacteria listed to Risk Group 1 (NBRC[1] & JCM[2]). The enzymes are important tool as food additives in food processing and required to be registered as food additives under the Ministry of Health, Labor and Welfare in Japan, and are therefore subject to strict safety guidelines. These guidelines are also applied to all microorganisms used for enzyme production. So, the esterase in this study is acceptable as food additive. The esterase purification procedure summarized in Table 1. The purified enzyme appeared as a single band on SDS-PAGE (data not shown). The final preparation of the esterase showed a 3.2-fold increase in specific activity over cell cultures on the basis of the standard assay method, with an overall yield of 0.5%. The molecular mass of the purified esterase was calculated to be 90.2 kDa from the result of SDS-PAGE.

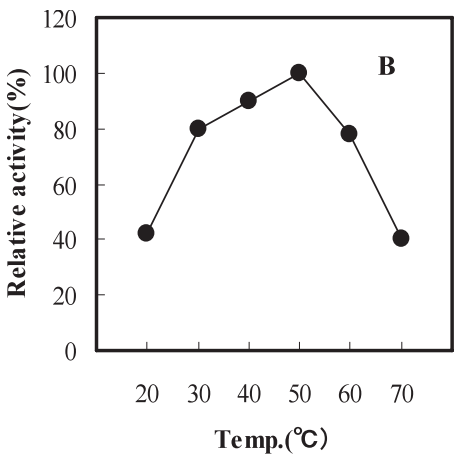
The purified enzyme showed maximum activity at pH 7.0 (Fig. 2-A) and 50°C (Fig. 2-B) and it was stable over a pH range of 5.0 to 8.0 (data not shown). The enzyme retained its original activity at temperatures below 50°C (Fig. 2-C), but was rapidly inactivated at temperatures higher than 60°C. Effect of chemicals on the esterase activity was measured at the standard assay conditions with 1 mM in the reaction mixture (Table 2). It is generally known that Ca^{2+} promote esterase activity, but did not promote this esterase activity. All chemicals inhibited this esterase activity. The flavors formation according to hydrolysis of milk fat in Condensed milk was examined by sensory evaluation. The milk flavors from reaction mixture were decreased rapidly. On the other hand, cheese flavors formed fairly strong (data not shown).

In conclusion, the esterase identified in this study from *B. ferrariae* L-2 is considered a suitable enzyme as a flavoring enzyme in dairy food processing.

Table 1. Purification of esterase.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture filtlate	342.65	4099	0.08	100
CM-Toyopearl 650M	113.07	6769	0.17	33.0
DEAE-Toyopearl 650M	62.89	326.0	0.19	18.4
Butyl-Toyopearl 650M	7.57	65.28	0.12	2.20
Toyopearl HW-55F	1.75	6.85	0.26	0.50





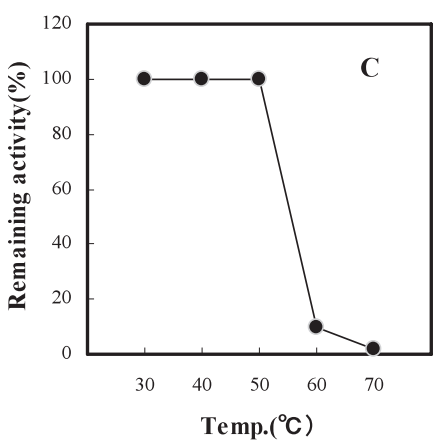


Fig. 2-A. Effect of pH on enzyme activity.

The enzyme activity was measured with standard assay method at several pH.
Mcllvaine buffer, pH 3-6; phosphate bufffer, pH 6-8; Atkins buffer, pH 9.

Fig. 2-B. Effect of temperature on enzyme activity.

The enzyme activity was measured with standard assay method at several temp.

Fig. 2-C. Thermalstability on enzyme activity.

After incubation at several temp. for 20 min, the remaining activity was assayed with standard assay method.

Table 2. Effect of chemicals on esterase activity.*

Chemical compaund	Relative activity (%)
none	100
Sodium Dodecyl Sulfate	78
EDTA	86
p-Chloromercuribenzoic acid	82
Monoiodoacetic acid	85
NaCl	119
$MgCl_2$	110
$FeCl_2$	95
$FeCl_3$	105
KCl	102
$CuCl_2$	94
$CaCl_2$	93
$CoCl_2$	109
$ZnCl_2$	111
$AlCl_3$	109
AgCl	120

* The esterase activity was measured by standard assay method with 1 mM chemicals. The relative activity was defined as a percentage of enzyme activity without chemicals.

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