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Isolation and biochemical characterization of two lipases from a metagenomic library of China Holstein cow rumen

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ABSTRACT

Two novel lipase genes *RlipE1* and *RlipE2* which encoded 361- and 265-amino acid peptides, respectively, were recovered from a metagenomic library of the rumen microbiota of Chinese Holstein cows. A BLAST search revealed a high similarity (90%) between *RlipE2* and a carboxylesterase from *Thermosinus carboxydivorans* Nor1, while there was a low similarity (below 50%) between *RlipE1* and other lipases. Phylogenetic analysis indicated that RlipE2 clustered with the lipolytic enzymes from family V while RlipE1 clustered with six other putative bacterial lipases which might constitute a new subfamily. The recombinant lipases were thermally unstable and retained 60% activity over a pH range of 6.5–8.5. Substrate specificity assay indicated that both enzymes had higher hydrolytic activity toward laurate (C_{12}), palmitate (C_{16}) and stearate (C_{18}). The novel phylogenetic affiliation and high specificity of both enzymes for long-chain fatty acid make them interesting targets for manipulation of rumen lipid metabolism.

Introduction

Ruminant products make an important contribution to the human diet but have caused concern due to their enriched saturated fatty acid content. Ruminant animals are characterized by a pregastric fermentation system which results in extensive transformations of ingested feedstuffs before they reach the duodenum. Previous studies have identified that dietary lipids undergo extensive hydrolysis and biohydrogenation in the rumen [1]. Lipids are readily hydrolyzed to the extent of 85% with the liberation of free long-chain fatty acids, a high proportion of which are unsaturated [2,3]. The extensive biohydrogenation of dietary unsaturated fatty acids in the rumen results in stearic acid as the major fatty acid entering the duodenum. Both the hydrolysis and biohydrogenation processes are accomplished almost entirely by rumen microbes [4,5]. The composition of the rumen microbial community is phylogenetically complex yet highly host-specific, consisting mainly of archaea, ciliate protozoa, bacteria, and fungi [6–8]. The application of 16S rRNA gene-based methods during the last decades has led us to realize that rumen microbial community is much more diverse and complex than ever suspected [6,9]. However, due to the fact that only a small proportion of ruminal bacteria have been cultured [10], little is still known about the physiological properties and metabolic pathways of rumen microorganisms involved in lipid metabolism. In contrast, direct cloning and sequencing of metagenomic DNA from environmental samples provides access to the genetic and metabolic potential of uncultured microorganisms that contribute to rumen metabolism. This approach, referred as environmental genomics or metagenomics, has been applied to explore new catalytic enzymes from a range of environments [11–15].

To recover genes associated with lipase activity in rumen, we have constructed a bacterial artificial chromosome (BAC) metagenomic libraries from rumen samples collected from Chinese Holstein cows. The BAC metagenomic library consisted of 15,360 clones. Restriction analysis revealed a high level of diversity of the cloned DNA fragments, and the average size of inserts was approximately 54.5 kb [16]. In the present study, two novel lipase genes were identified using a selective screening assay for lipolytic activity from this BAC library, followed by the biochemical characterization of the purified protein.

Materials and methods

Screening of the genomic library. Transformants from the library were screened onto Luria–Bertani agar plate containing 1% (w/v) trioleoylglycerol plus 0.001% Rhodamine B dye. Rhodamine B was dissolved in distilled water and sterilized by filtration. LB agar medium containing 1% arabic gum was autoclaved and cooled to approximately 60 °C. Trioleoylglycerol and Rhodamine B were added and emulsified by mixing with a homogenizer. After the medium was allowed to stand for 10 min at 60 °C and thus reduce foaming, 20 ml of the medium was poured into each Petri dish. Transformed cells were spread on the plate and incubated at

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37 °C for 2 d. Lipase production was monitored by irradiating plates with UV light at 350 nm [17].

Whole BAC sequencing. Two whole BAC clones were entirely sequenced by Beijing Genomic Institute as follows: DNA manipulations were carried out according to standard procedures [18]. Mechanically sheared DNA of the BAC was ligated to pBluescript SK- for shotgun sequencing. DNA sequencing reactions were analyzed using an ABI-3100 automated sequencer (Applied Biosystems, USA). After sequence assemblage, the gap was closed by the method of primer-walking.

Gene annotation and analysis of sequence features. GC composition was studied with the GC Content Grapher (http://plantst.sdsc.edu/plantst/html/geneGC.shtml). Open reading frameworks (ORFs) were characterized using the ORF-finder software at the NCBI (www.ncbi.nlm.nih.gov/gorf/gorf.html). Several criteria were used to define ORFs among all the potential ORFs detected. First, only sequences encoding peptides longer than 50 amino acids, preferentially non-overlapping, were retained. Second, when putative ORFs were detected in different reading frames, we selected those that had known homologs and third, in the case of various putative overlapping ORFs in different reading frames with no known homologs, we selected the ORFs that had the longest sequence. We used the programs PSI-BLAST, TBLASTN and BLASTP to look for sequence similarity in each ORF product with published protein sequences [19]. Predicted proteins were classified according to the Clusters of Orthologous Groups of proteins (COGs) database (www.ncbi.nlm.nih.gov/COG/) [20]. Amino acid alignments were performed with the CLUSTAL W program [21]. Phylogenetic analysis was conducted using the program MEGA [22].

Recombinant expression and protein purification. The ORF of the putative lipase gene was amplified by PCR and inserted into pET-22b(+) expression vector (Novagen, USA). The recombinant plasmids were used for the expression of the lipase genes in *Escherichia coli* strain BL21 (DE3). The transformed cells were grown at 37 °C in 500 ml LB medium containing 25 μg ml^{-1} kanamycin. When the cultures reached an optical density of 0.5 at 600 nm (OD_{600}), they were induced for 4 h with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG).

Cells were harvested by centrifugation at 6000*g* for 10 min and washed with 50 mM Tris–HCl buffer (pH 8.0). The cell pellet was re-suspended in the same buffer, and the cells were then disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation at 15,000*g* for 30 min. The recombinant lipase in the insoluble form was denatured with 6 M urea and then refolded by fractional dialysis in 3, 1.5, and 0 M urea in 50 mM Tris–HCl (pH 8.0). The target protein was purified by loading the crude enzyme fraction on a Ni^{2+}-immobilized Chelating Sepharose Fast Flow (Pharmacia Biotech, Sweden). The eluted solution samples showing lipase activity were pooled and concentrated by ultra-filtration using a YM10 membrane (Millipore, USA).

Lipase activity. Lipase activity was measured spectrophotometrically. The reaction mixture consisted of 0.01 ml of 10 mM *p*-nitrophenyl palmitate (*p*NPP) as a substrate in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM Tris–HCl buffer (pH 8.0) containing an appropriate amount (10 μl) of the enzyme. The enzyme reaction was performed for 5 min at 25 °C. The amount of *p*-nitrophenol liberated during the reaction was measured by absorbance at 405 nm. One lipase unit was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute.

Substrate specificity of lipase enzyme was assayed using various *p*-nitrophenyl esters with acyl chains of different lengths (butyrate, C_4; valerate, C_5; caproate, C_6; octanoate (C_8); caprate, C_{10}; palmitate, C_{16}, and stearate, C_{18}) under standard assay conditions. Substrate specificity was also examined by titrating free fatty acids liberated from tributyrin (C_4), tricaprylin (C_8), and triolein ($C_{18:1}$ [*cis*-9]). The assay mixture, containing 0.5 ml of triacylglycerols, 5 ml of acetate buffer (pH 5.6), 10 mM $CaCl_2$, and enzyme (2 μg/ml), was incubated at 30 °C for 30 min with magnetic stirring at 500 rpm. The enzyme reaction was stopped by the addition of 2 ml of ethanol. The amount of fatty acids released during the incubation was determined by titrating the mixture with 50 mM KOH to pH 10.0 using an Orion 960 titrator (Thermo Scientific, USA). One unit of lipase activity was defined as the activity required to release 1 μmol of fatty acids per min under the above conditions.

The optimum temperature and pH for enzyme activity were determined spectrophotometrically with *p*-NPP as a substrate. The assay was performed by incubation of the reaction mixture over a temperature and pH range of 0–70 °C and 4–11, respectively.

Nucleotide sequence accession number. The sequences of Rlip1 and Rlip2 were deposited in GenBank under Accession Nos. FJ529693 and FJ529694.

Results and discussion

Screening and sequence analysis

Screening for lipase activity of the whole BAC library yielded eighteen positive clones. Two clones which showed the highest lipolytic activity (largest halos) were designated as Rlip1 and Rlip2, and were used for further analysis.

The inserts of the two BAC clones were sequenced completely in order to identify the genes encoding lipase activity. The insert sequences of clones Rlip1 and Rlip2 were 25,396 and 10,979 bp, had 50.7% and 57.1% G+C content and contained 23 and 11 predicted ORFs, respectively. Most of the predicted protein-coding genes of Rlip1 and Rlip2 (15 of 23 and 7 of 11, respectively) showed significantly similarity to genes of known function, some (3 of 23 ORFs and 2 of 11 ORFs for Rlip1 and Rlip2, respectively) were conserved hypothetical genes, and other ORFs (5 of 23 ORFs and 2 of 11 ORFs for Rlip1 and Rlip2, respectively) did not show any similarity to sequences in the database. The protein coding regions of Rlip1 were not closely related to those of a known bacterial group. The most closely related homologs found in BLAST search for 23 ORFs from Rlip1 resulted in affiliations with 14 different organisms (Table 1). In contrast, the majority of the coding region (64%) of Rlip2 showed typically $<e^{-11}$ *E*-value to *Thermosinus carboxydivorans* ORF homologs (Table 2).

One of the ORFs from Rlip1, which is designated *RlipE1*, encoded a 361-amino-acid putative lipase. *RlipE1* contains the lipase-conserved catalytic nucleophile Ser244 in the consensus pentapeptide GLSFG, which matches the characteristic Gly-X-Ser-X-Gly motif found in lipolytic enzymes [23]. Similarly, an ORF from Rlip2 which encoded a 225-amino-acid putative lipase was named as *RlipE2*. The consensus pentapeptide GLSMG was found in Ser107.

A BLAST search of GenBank revealed a relatively low similarity (below 50%) between *RlipE1* and other bacterial esterases/lipases, including an esterase (EDY81893 from *Verrucomicrobiae bacterium* DG1235 (48%), a lipase (ZP_02066438) from *Bacteroides ovatus* (41%), a glycoside hydrolase (YP_001297384) from *Bacteroides vulgatus* ATCC 8482 (43%), an esterase (ZP_02737217) from *Gemmata obscuriglobus* UQM 2246 (42%), an esterase (YP_592320) from *Acidobacteria bacterium* Ellin345 (41%) and a acetyl xylan esterase (CAJ19122) from an unidentified microorganism (38%). None of these bacterial lipases have been characterized. Arpigny and Jaeger [23] previously reported the extensive classification of bacterial lipolytic enzymes based on a comparison of their amino acid sequences. To classify the phylogenetic position of RlipE1, we selected 50 bacterial lipolytic enzymes representing eight different families and constructed a phylogenetic tree. As shown in Fig. 1, RlipE1 and six other putative bacterial lipases clustered together