

Cloning and Characterization of a Novel α -Amylase from a Fecal Microbial Metagenome^S

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To isolate novel and useful microbial enzymes from uncultured gastrointestinal microorganisms, a fecal microbial metagenomic library of the pygmy loris was constructed. The library was screened for amylolytic activity, and 8 of 50,000 recombinant clones showed amylolytic activity. Subcloning and sequence analysis of a positive clone led to the identification a novel gene (*amyPL*) coding for α -amylase. AmyPL was expressed in *Escherichia coli* BL21 (DE3) and the purified AmyPL was enzymatically characterized. This study is the first to report the molecular and biochemical characterization of a novel α -amylase from a gastrointestinal metagenomic library.

Keywords: Amylase, metagenome, function-based screening, gastrointestinal microorganisms, cloning, characterization

Gastrointestinal microbes possess numerous hydrolases that mediate hydrolysis and subsequent fermentation of ingested foods. Thus, the intestinal microbiome has been recognized as a rich source of enzymes. However, various molecular analyses have confirmed that >99% of microorganisms cannot be cultured by conventional methods [17]. This unexplored microbial diversity represents an untapped source of potentially novel and unique enzymatic activities and metabolic pathways. To search for new or enhanced enzymes from unculturable intestinal biota, total gut microbial metagenomes have been extracted to construct metagenomic libraries or metagenomic shotgun sequences. Previous studies have shown that this strategy generates a large pool of novel hydrolases, such as lipases/esterases [4, 5, 23], amylases [4, 18, 20, 24], cellulase [3, 6, 14, 21],

xylanase [14, 15, 24], and glucosidase [1, 7], from the gastrointestinal tracts of humans and animals. These studies have provided further evidence that the gut microbiome is a rich source of glycosyl hydrolases containing currently unculturable microbes. Previous sequence-based metagenomic analyses have revealed the extensive diversity of glycoside hydrolase (GH) families in pygmy loris feces [22]. Thus, this study aims to search for hydrolases in a fosmid library constructed from pygmy loris fecal samples.

In this present study, we describe the construction of a metagenomic library and function-based screening of this library for amylase. One novel amylase gene (*amyPL*) has been identified, recombinantly expressed, and characterized from the constructed library.

Fresh fecal samples from pygmy loris were collected

from the Daweishan Nature Reserve of Pingbian, Yunnan Province, China. High-molecular-weight DNA extraction from the fecal samples was performed using the protocol previously described by Morita *et al.* [13]. A metagenomic library containing 2.36×10^5 clones was constructed using a CopyControl Fosmid Library Production Kit (Epicentre, USA), according to the manufacturer's instructions. The library was replicated in agar plates containing 0.1% (w/v) soluble starch for detecting amylase activity [18]. From a screening of 50,000 clones, we identified 8 that exhibited amylase activity.

The most active amylase-positive fosmid clone was subcloned into the pUC118 vector and screened for amylolytic activity. Positive clones were sequenced, and the open reading frames (ORFs) were identified by ORF Finder (Open Reading Frame Finder, <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Sequence manipulation was performed using Vector NTI 11.5.1 software (InforMax, USA). The signal sequence was predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>). It suggested that a ~1.5 kb DNA fragment derived from this clone encodes a gene possessing amylase activity. The *amyPL* gene was

1,539 bp long, and the molecular mass of the translated protein was estimated to be 55.4 kDa. The deduced AmyPL polypeptide was 512 amino acid residues in length, including the first 27 residues forming a putative signal peptide (Fig. S1). The cleavage site of the signal peptide was predicted between A27 and A28. Seven conserved regions of the GH13 α -amylase family [9] and catalytic triad [10] were identified in AmyPL. The catalytic triad consisted of Asp233 (catalytic nucleophile at β_4), Glu265 (proton donor at β_5), and Asp336 (transition-state stabilizer at β_7).

Homologs of AmyPL were obtained by searching the NCBI protein database using the PSI-BLASTP program (<http://www.ncbi.nlm.nih.gov/BLAST/>). AmyPL exhibited the highest identity (70%) to the uncharacterized putative protein (CDC15165) of *Bifidobacterium pseudocatenulatum* derived from a human gut metagenome, followed by 68% identity to the hypothetical protein (EDN82501) of *B. adolescentis*, 64% identity to the hypothetical protein (EEP20324) of *B. angulatum*, and 61% identity to the putative α -amylase of *B. gallicum*. Many of these putative proteins were revealed by whole-genome sequencing, but none has been biochemically characterized.

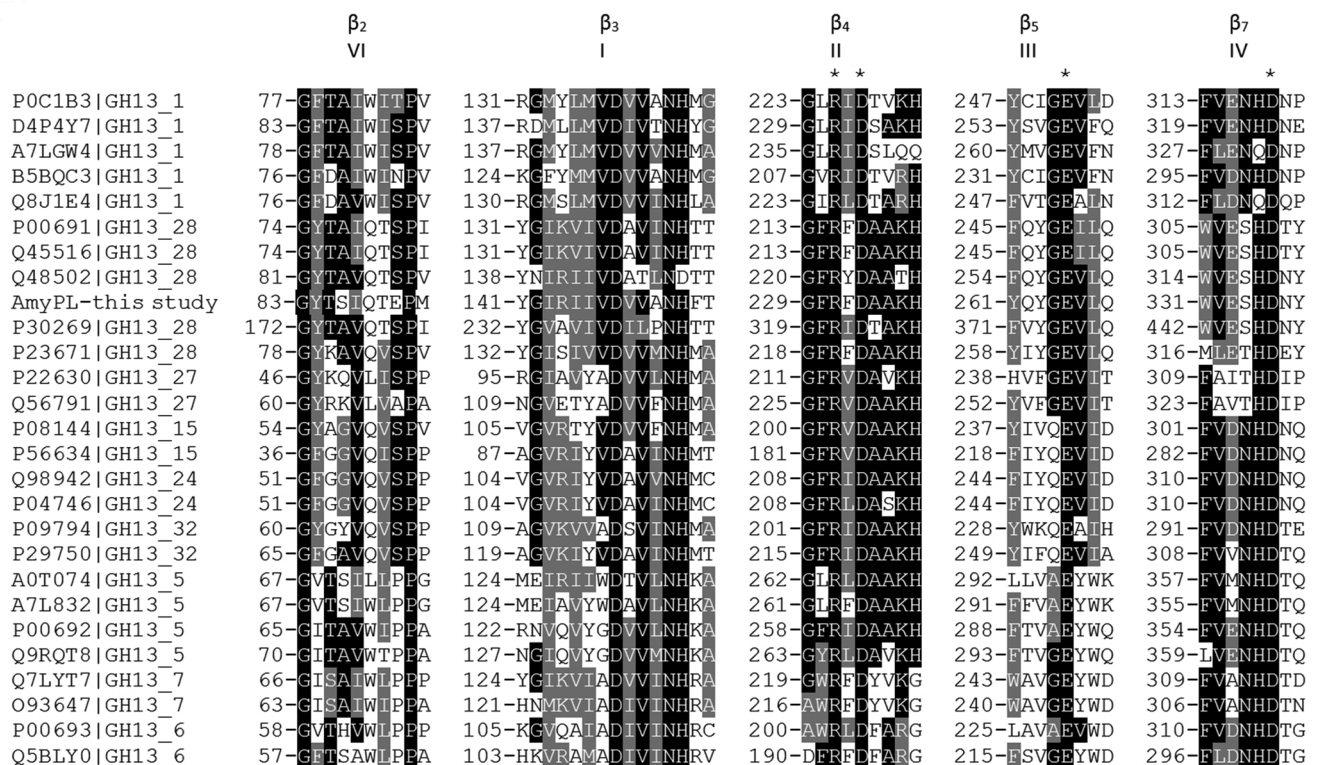


Fig. 1. Partial alignment of the amino acid sequences of AmyPL and the family GH13 α -amylase proteins.

Highly conserved residues are boxed in black. Four invariant residues are signified by asterisks.

Based on the previously reported classification of α -amylases in GH13 [2, 8, 16, 19], we chose 26 amino acid sequences of known α -amylases to represent the 9 established subfamilies. These proteins, together with AmyPL, were used for analyzing the sequence features (Fig. 1) and determining the phylogenetic tree (Fig. 2). Alignment of the amino acid sequences of AmyPL with other enzymes belonging to the GH13 α -amylase family revealed that AmyPL had five conserved regions and four amino acid

residues necessary for activity in the α -amylase family [11] (Fig. 1). The evolutionary tree clearly illustrates the clustering of AmyPL within the α -amylase subfamily GH13_28 (Fig. 2). The nucleotide sequence of AmyPL was deposited in the GenBank database under Accession No. KF311768.

The gene was amplified by PCR using the primers in Table S1. The PCR product was cloned into the pEASY-E1 expression vector (TransGen, China). The recombinant

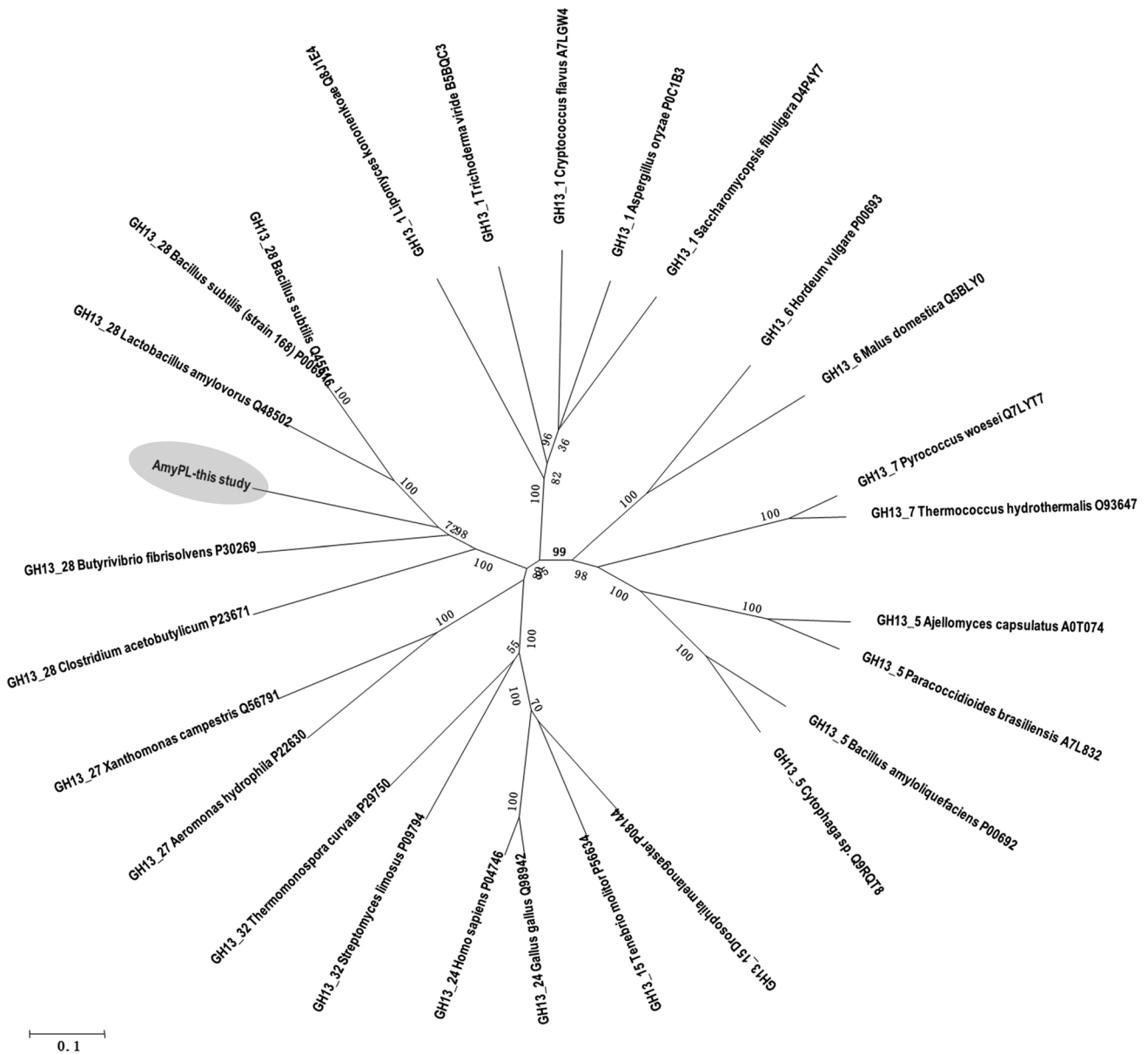


Fig. 2. Neighbor-joining phylogenetic tree of the GH13 α -amylase family. The tree was constructed using the neighbor-joining method with 1,000 bootstrap replicates using MEGA 4.0. The individual α -amylases are represented by the GH13 subfamily number, species, and Uniprot accession numbers.

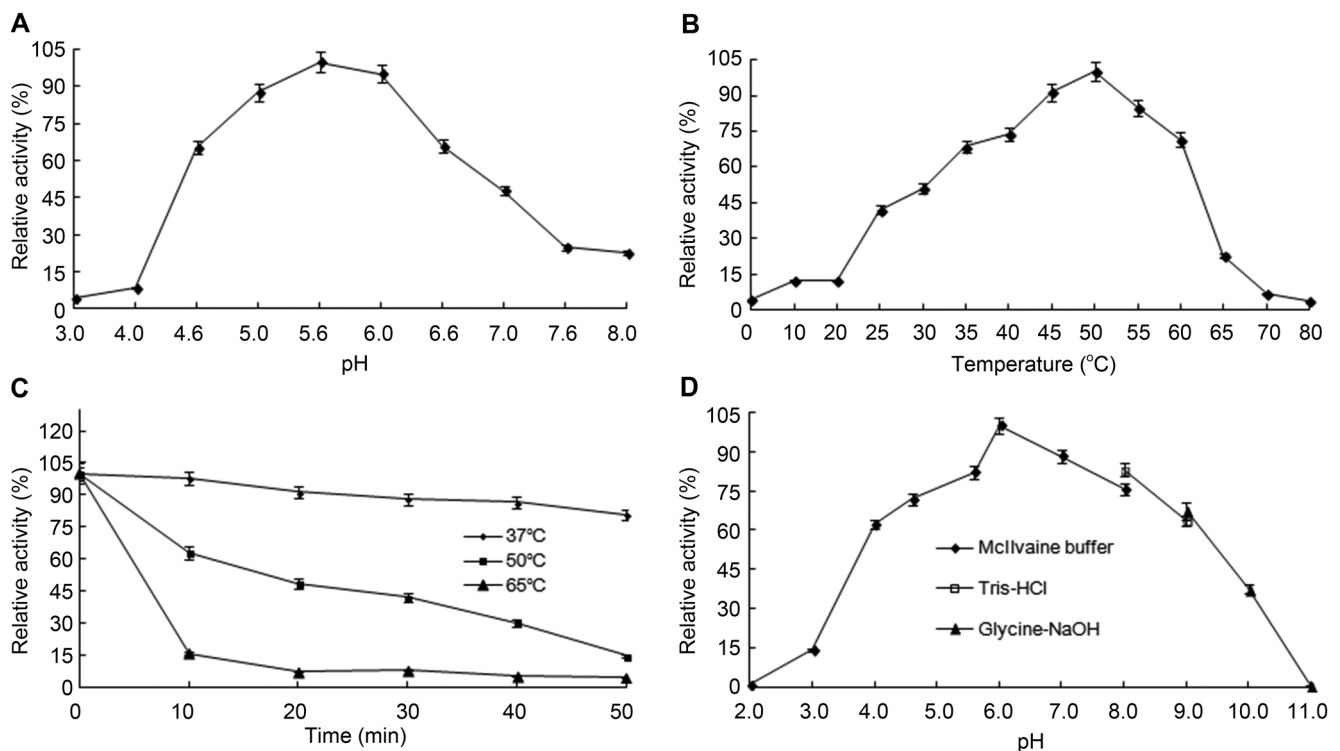


Fig. 3. Characterization of purified AmyPL.

(A) Effect of pH on AmyPL. The assay was performed at 50°C in buffers at pH 3.0 to 8.0. (B) Effect of temperature on AmyPL measured in Mcllvaine buffer (pH 5.6). (C) Thermostability of AmyPL. The enzyme was pre-incubated at 37°C, 50°C, or 60°C in Mcllvaine buffer (pH 5.6), and aliquots were removed at specific time points for the measurement of residual activity at 50°C in Mcllvaine buffers (pH 6.5). (D) pH stability of AmyPL. After pre-incubating the enzyme at room temperature for 1 h in pH 2.0–11.0, the activity was measured at 50°C in Mcllvaine buffers (pH 6.5).

plasmid (pEasy-E1-AmyPL) was then transformed into Trans1-T1-phage-resistant chemically competent cells (TransGen, China). Forward positive clones were screened by PCR using vector primer T7 and primer plAR (Table S1), and further confirmed by nucleotide sequencing. The valid recombinant plasmid (pEasy-E1- AmyPL) was transformed into *E. coli* BL21 (DE3) competent cells. Induction of the recombinant enzyme and enzyme purification were performed as previously described by Zhou *et al.* [25]. The molecular mass of the purified enzyme was close to the calculated value of AmyPL (55.4 kDa) (Fig. S2).

The α -amylase activity was determined by measuring the amount of reducing sugar using 3,5-dinitrosalicylic acid [12]. Through an assay using soluble starch as the substrate, purified recombinant AmyPL showed optimal α -amylase activity at pH 5.6, and retained >65% of the maximum activity between pH 4.6 and 6.6 (Fig. 3A). The thermal activity of purified AmyPL was optimal at 50°C when assayed at pH 5.6, and >65% of the maximal activity was

retained when assayed at 35°C to 60°C (Fig. 3B). The enzyme was stable and retained >80% activity after incubation at 37°C for 50 min (Fig. 3C). Under its optimal temperature, AmyPL lost approximately half of its activity after 20 min of incubation. Purified AmyPL exhibited >70% of the initial activity after incubation in buffers ranging from pH 4.6 to 8.0 at room temperature for 1 h (Fig. 3D). The activity of purified AmyPL was completely inhibited by 10 mM Hg^{2+} , and partially inhibited (retaining ~80% activity) by 10 mM Fe^{3+} and Pb^{2+} (Table S2). β -Mercaptoethanol and Co^{2+} enhanced the activity.

The hydrolysis pattern of soluble starch digested with purified AmyPL was analyzed by thin-layer chromatography (TLC) on 0.2 mm silica gel plates with a solvent system consisting of *n*-butanol, glacial acetic acid, and water (4:3:2 (v/v)). Sugars were detected by heating the TLC plates at 110°C for 10 min after dipping the plates in diphenylamine-aniline reagent (50 ml of acetone, 1 ml of aniline, 1 g of diphenylamine, and 5 ml of 85% phosphoric acid; stock 4°C)

(Fig. S3). Maltose and maltotriose were formed in great amounts at the early stage of hydrolysis. On further incubation, maltotriose subsequently hydrolyzed to accumulate glucose and maltose.

In this study, a total of eight amylolytic-active positive fosmid clones were screened using an activity-based method from a fecal microbial metagenomic library of pygmy loris, and a novel α -amylase gene, *amyPL*, was identified. To the best of our knowledge, AmyPL is the first α -amylase isolated from a gastrointestinal metagenomic library that has been biochemically characterized. This study contributes to knowledge of the diversity of amylolytic genes and demonstrates that gut microorganisms are a very important source of novel hydrolase genes. Future studies should include further cloning and characterization of the other amylolytic-active positive clones detected in this study and screening of other hydrolases from this metagenomic library.

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