Cloning of α-Amylase Gene from Unculturable Bacterium Using Cow Rumen Metagenome

Soo Jeong Cho and Han Dae Yun[1,2]*

Laboratory of Microbial Functions, Bioprocess Engineering Division, Korea Research Institute of Bioscience and Biotechnology, Yusong, Daejeon 305-3330, Korea, [1]Division of Applied Life Science, Gyeongsang National University, Chinju 660-701, Republic of Korea, [2]Research Institute of Agriculture & Life Sciences, Gyeongsang National University, Chinju 660-701, Republic of Korea

Received August 12, 2005 / Accepted December 14, 2005

The metagenomes of complex microbial communities are rich sources of novel biocatalysts. The gene encoding an extracellular α-amylase from a genomic DNA of cow rumen was cloned in *Escherichia coli* DH5α and sequenced. The α-amylase (*amy*A) gene was 1,893 bp in length, encoding a protein of 631 amino acid residues with calculated molecular weight of 70,734 Da. The molecular weight of the enzyme was estimated to be about 71,000 Da by active staining of a SDS-PAGE. The enzyme was 21 to 59% sequence identical with other amyloyltic enzymes. The AmyA was optimally active at pH 6.0 and 40℃. The AmyA had a calculated pI of 5.87. AmyA expressed in *E. coli* DH5α was enhanced in the presence of Mg^{2+} (20 mM) and Ca^{2+} (30 mM) and inhibited in the presence of Fe^{2+} and Cu^{2+}. The origin of *amy*A gene could not be confirmed by PCR using internal primer of *amy*A gene from extracted genomic DNA of 49 species rumen culturable bacteria so far. An *amy*A is supposed to obtained from unculturable rumen bacterium in cow rumen environment.

Key words – Rumen metagenome, *amy*A, Unculturable bacterium, Starch-SDS-PAGE

Modern biotechnology has a steadily increasing demand for novel biocatalysts, which has prompted the development of novel experimental approaches to find and identify novel biocatalyst-encoding genes. Recently, the studies were initiated to investigate the metagenome[10,23]. Several different laboratories have successfully isolated novel genes encoding different enzymes and secondary metabolites from microbial communties and their metagenomes without cultivation of the microbes[3,4,7,13,17]. The microbial niches were studied highly diverse and ranged from moderate environments, such as river soil[12], to rather extreme environments, like the deep sea and lake [2,22].

The rumen ecosystem comprises a diverse population of obligately and anaerobic bacteria, fungi, and protozoa defined by the intense selective pressures of the ruminal environment[29-31,33,36]. The rumen microbial population presents a rich and, until recently, under utilized source of novel enzymes with tremendous potential for industrial application. The enzyme activities confirmed to exist in the rumen are diverse, and include plant cell wall polymer degrading enzymes (e.g. cellulase, xylanase, β-glucanase, and pectinases), amylase, protease, phytases, and specific plant toxin-degrading enzymes (e.g. tannases). The variety of enzymes present in the rumen arises not only from the diversity of the microbial community but also from the multiplicity of fibrinolytic enzymes produced by individual microorganisms[28].

Starch is the major carbohydrate of many plants and enzymes digesting starch are widely distributed in nature. The α-amylase (1, 4-α-D-glucanohydrolase; EC 3.2.1.1) are which hydrolyzes α-1, 4 linkage in starch-related molecules, is one of several enzymes involved in starch degradation. They are among the most important commercial enzymes, having wide applications in starch-processing, brewing, alcohol production, textile and other industries. Numerous α-amylases from eubacteria, fungi, plants, and animals have been characterized, and their genes have been cloned.[7,18,33]. Walker reported the existence of hydrolytic starch-degrading activities in *Streptococcus bovis*[34]. After, Some researchers reported the extra and intracellular α-amylase gene in *Streptococcus bovis* [5,6,25,26,35].

Before this study, our researchers reported on the microorganisms diversity in cow rumen[29-31]. Also, we first reported on the cloning and sequencing of the α-amylase gene from unculturable bacteria using cow rumen metagenome.

***Corresponding author**
Tel : +82-55-751-5499, Fax : +82-55-757-0178
E-mail : hdyun@nongae.gsnu.ac.kr

Materials and methods

Bacterial strains and growth conditions

Rumen bacteria (Table 1) were cultured as ATCC and DSMZ recommended medium, respectively. *E. coli* DH5α, *E. coli* EPI300™, and recombinant *E. coli* cells were cultured in LB (Difco, USA) containing appropriate antibiotics (ampicillin, 50 μl/ml; chloramphenicol, 12.5 μl/ml) at 37℃.

Sampling of rumen metagenome

Samples of rumen content were obtained from a closed herd at the Chinju National University (Chinju, Korea). The animals were rumen-fistulated Korean cows (HANWOO) with the body weight 400±10 kg, fed a mixed ration (rice hulland concentrate in a 4:1 ratio) twice a day. The concentrate was purchased from Daehan Food (Ulsan, Korea). Representative samples of total rumen contents were collected from the animal via the ruminal fistula before the morning feeding. The samples on ice were immediately transferred into an anaerobic box and stored at -80℃.

Construction of cow rumen metagenomic library

A genomic library was constructed in the fosmid vector pCC1FOS™ as previously described[17]. Genomic DNA from cow rumen was sheared into approximately 40 kb fragments using a syringe needle, size fractionated on a 5 to 40% linear sucrose gradient and then end-repaired to yield blunt, 5'-phosphorylated ends. The resulting DNA fragments were ligated with the cloning ready pCC1FOS™ vector, and then packaged using a lambda DNA packing kit (Epicentre, USA). The library was screened on LB media, containing starch for amylase. One clone (pCHJ2) bearing amylase activity was isolated.

Recombinant DNA techniques

Standard procedures for restriction of endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning related techniques were followed as described by Sambrook *et al*[24].

Cloning and DNA sequencing of *amyA* gene

For subcloning, pYCHJ2 was partially digested with *Sau*3AI (Promega, USA). Two to five kb fragments of the cosmid DNA from this partial digestion were ligated into the *Bam*HI (Promega, USA) site of pBluescript II KS+ (Stratagene, USA) vector treated by CIP (Promega, USA), and then transformed into *E. coli* DH5α. One positive subclone (pYCHJ100) was obtained. To detect bacterial colonies were grown on a amylase indicator medium [LB agar plates containing appropriate antibiotics and 1% soluble starch (Sigma, USA)]. After growth at 37℃ for 24 hr, the plate was stained with 0.5% I_2-KI solution for 30 min. Active band appeared as a white halo on a dark blue background. Nucleotide sequences were determined by the dideoxy chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., USA). Assemly of the nucelotide sequences and the amino acid sequences analysis were performed with the DNAMAN analysis system (Lynnon Biosoft, Canada). The BLAST program was used to find the protein coding regions.

Amylase assay

The α-amylase activity in the recombinant *E. coli* clones was determined by measuring the amount of reducing sugars during incubation with starch. A selected volume of enzyme was diluted with 10 mM Tris-HCl buffer (pH 7.0) to a total volume of 0.5 ml and was added to 259 μl of 1% soluble starch dissolved in 10 mM Tris-HCl buffer (pH 7.0), and the mixtures were incubated at different temperatures for 20 to 60 min. One unit of the enzyme that liberted 1 μmol of reducing sugar per min at 70℃. The amounts of reducing sugars released were determined by the dinitrosalicylic acid method[20].

AmyA active staining after Starch-SDS-PAGE

Starch-SDS-PAGE was performed as described by the method of Lim *et al*[18]. *E. coli* cells harboring the amylase gene were cultured at 37℃ for 24 hrs in LB medium supplemented with 0.1% soluble starch. Whole cell extracts from these cultures were prepared by sonication (three times for 30 sec each at 4℃). The cell extracts and supernatants were mixed 1:1 (v/v) with sample buffer (62 mM Tris-HCl pH 6.8, 10% glycerol, 0.025% bromophenol blue, 5% β-mercaptoethanol, and 2% SDS), heated at 95℃ for 3 min. The protein samples (30 μg) were separated by SDS-PAGE (BioRad, USA). Subsequently the protein was renatured by incubation in three changes of 250 ml of 10 mM Tris-HCl (pH 7.5), 1% Triton X-100 with shaking for 12 hrs. Finally the gel was incubated in 10 mM Tris-HCl (pH 7.5) at 40℃ for 8 hrs. The gel was stained with a 0.5% I_2-KI solution for 30 min. Active band appeared as white halo on a dark background.

Results

Detection of α-amylase positive clone and subcloning

The amylase gene was isolated from a library of cow rumen metagenome. The clone showed the white ring that indicated α-amylase activity. The clone was found to contain an insert of 7.0 kb. The pBluescript II KS+ containing 3.6 kb insert, pCHJY100, was further characterized (Fig. 1). Analysis of subclones of pCHJY100 revealed that the 3.1 kb *Eco*RI fragment in pCHJY110 was sufficient to confer amylase activity in the plate assay (Fig. 1).

Sequencing and sources of *amy*A

The entire 3.6 kb insert in pCHJY100 was sequenced in both strands, and an ORF was identified (Fig. 2). An *amy*A was 1,893 bp in size and encodes a protein of 631 amino acids with a predicted molecular mass of 71,000 Da. The ORF *amy*A starts the ATG initiation codon and the reading frame ends with the opal stop codon TGA at position 2,526 (Fig. 2). The N-terminal 23 amino acids of *amy*A separated from the rest of the protein by a potential cleavage site in front of Lys_{24} have the typical features of a prokaryotic signal peptide (Fig. 2). The N-terminal amino acid sequences of the protein eluted from SDS-PAGE gel closely matched the amino acid residues 1 through 5 of the translated sequences

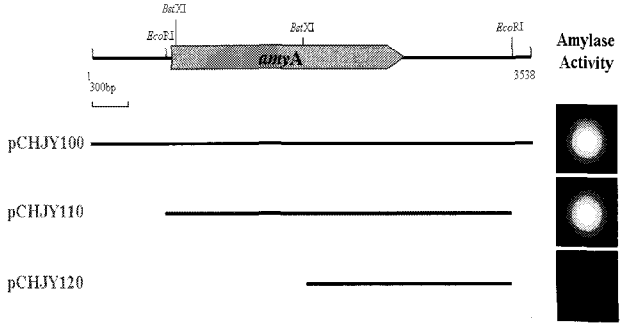

Fig. 1. Physical map of the *amy*A gene from rumen metagenomic library and detection of an amylase gene by starch agar diffusion method. The cells were incubated at 37℃ for 48 hr. The cleavage sites of restriction enzymes *Eco*RI and *Bst*XI are shown.

```
1     CCCTGCAGGGCGGAGACGGGGTTGATGAGGTTACCGTTGAGTTCCTTCGGCCTTGATCCCCATGGAGGAAAGCTCAAACGCATCTTCCAGTACCGGGTTCCACACCAGAATATCGCCGTT
121   CAGACCCGCATGGCCCAGCTCTGACGGGGTGCTCCAGTCATCATAATCCGGTGCGCGCACGTCGTGGCGATGACCATCGCTCAGCTTGCCGCCAATCCCGACAAGGAATACCGCGCCAAG
241   ATCTACCACGCCCGGCATGTCGTAGGTGTTGCCTTGGTACTCCACCTTGTTTGGAGGCAACATGTCGCCGGTAATCTGCACTTTCTGAGCCTCTGGCGCAATCAAATTAAACGTCACGGA
361   ATTGTCTGCATGCACATCAGGTGAAACCACCTGCGGTCCCTGTCCCCAGTTCAGATTCTGCTGGGCCATTGCTGCCATACCGACCAAGCAGAGGGCAGCCGTAAGGAATAGTTTTTTCAT
401   ACTGTTGTTTGTTTTTAGTTGTTATAGTGCTGCAAATATACACAAATTTTTGAAAACACAAAGAATCTCACGCATATTTCTATGCCGAATGAAAAATATTTCGTAACTTCGCCGCGGAA
601   TTCTATGAAATAATGATTAAACTTAAATCGATATGAGAAAACTATTATTGTTAACCGCCATGCTGATGGCAAATGTAATGATGATGAATGGAGCAAAAGTAAAAGTGACTGTAGACCGTG
1                                  M  R  K  L  L  L  L  T  A  M  L  M  A  N  V  M  M  M  N  G  A  K  V  K  V  T  V  D  R
                                                                  Putative signal peptide
721   TGGATCCCACCGACTGGTATGTGGGCATGAAGAATCCCTCGTTGCAGTTGATGGTGTATGGTAAGAACATTGCCAGTGTAAAAGATGTAACGACGGATTATCCTGGAGTGGTCATCGACA
30    V  D  P  T  D  W  Y  V  G  M  K  N  P  S  L  Q  L  M  V  Y  G  K  N  I  A  S  V  K  D  V  T  T  D  Y  P  G  V  V  I  D
841   GCATTGTAAGACTAGAGTCGCCCAACTACCTATTGGTGTATCTGAACCTGCGCGACGCACAGCCGGGTACGATGACATTGAAGTTGGACAAACTGAAAGTCAGCTATCTGTTGAAGCAGC
70    S  I  V  R  L  E  S  P  N  Y  L  L  V  Y  L  N  L  R  D  A  Q  P  G  T  M  T  L  K  L  D  K  L  K  V  S  Y  L  L  K  Q
961   GTGAGATGGCTGGTTCGAAGCGCATGGGATTTACCAATGCCGACGTACTTTACATGCTTATGCCAGACCGTTTTGCACAGGGTGCAGGGCATAATCCGCAGGTGGCGGGAATGCGAACGT
110   R  E  M  A  G  S  K  R  M  G  F  T  N  A  D  V  L  Y  M  L  M  P  D  R  F  A  Q  G  A  G  H  N  P  Q  V  A  G  M  R  T
1081  ATAAGGAAGACCGTACGCAGCCCTCATTACGACACGGTGGCGACCTGAACGGCATTCGCGAACATCTGGATTATTTCAACGAACTGGGCGTGACGGCGCTGTGGTTGACACCCGTGCTGG
150   Y  K  E  D  R  T  Q  P  S  L  R  H  G  G  D  L  N  G  I  R  E  H  L  D  Y  F  N  E  L  G  V  T  A  L  W  L  T  P  V  L
1201  AGAACGACTCGCCGGACGACGAACGCGGCTACTCCACCTATCACGGCTATGCCACGACGGACTACTATCGTGTTGACCCACGATTCGGCACGAACGAGGACTATCGCCGACTCTGCGATG
190   E  N  D  S  P  D  D  E  R  G  Y  S  T  Y  H  G  Y  A  T  T  D  Y  Y  R  V  D  P  R  F  G  T  N  E  D  Y  R  R  L  C  D
1321  AGGCTCATGCGAAGGGACTGAAGGTGGTGATGGACATGATTTTCAATCACTCTGGTTTCGAGCATCCCTGGACGCAGGACATGCTGACGAAGGACTGGCTCAACTTGCCCGACTGGCTGA
230   E  A  H  A  K  G  L  K  V  V  M  D  M  I  F  N  H  S  G  F  E  H  P  W  T  Q  D  M  L  T  K  D  W  L  N  L  P  D  W  L
                                     Region I
1441  AGGAATCGCAGGGAACGAGTAATCCGACAGGAACCTGTTTCCAGCAGACGAGCTACAAGCTGACGCCGGTCAAGGATCCCTACGCTTCGCAGGTTGACCTGCGCGAGACCGTGGACGGTT
270   K  E  S  Q  G  T  S  N  P  T  G  T  C  F  Q  Q  T  S  Y  K  L  T  P  V  K  D  P  Y  A  S  Q  V  D  L  R  E  T  V  D  G
1561  GGTTTGTACCCACCATGCCCGACCTGAACCAGCGCAATCCGCATGTGATGACCTATCTTATCCAGAACTCCAAATGGTGGATAGAGACCATCGGCATCGACGGCATTCGTATGGACACCT
310    W  F  V  P  T  M  P  D  L  N  Q  R  N  P  H  V  M  T  Y  L  I  Q  N  S  K  W  W  I  E  T  I  G  I  D  G  I  R  M  D  T
                                                                                                                  Region II
1681  ACCCGTATGCTGATGCCAAGGGTATGGCATGTTGGATGAAAGAATTGGACGAGGAATACCCCAACTTCAACACCGTCGGTGAGACGTGGGTCACGGAGCCTGCCTATACCGCCGCGGTGGC
350   Y  P  Y  A  D  A  K  G  M  A  C  W  M  K  E  L  D  E  E  Y  P  N  F  N  T  V  G  E  T  W  V  T  E  P  A  Y  T  A  A  W
                                                                  Region III
1801  AGAAGGACTCAAAGCTGTCGAAGGAGAACTCGTATCTGAAGACCGTTATGGACTTCTCGTCTTCGAAAAGCTGTCGCAGGCAAAGAACGAGGAAACCGACGCTTGGTGGCAGGGACTGAT
390   Q  K  D  S  K  L  S  K  E  N  S  Y  L  K  T  V  M  D  F  S  F  F  E  K  L  S  Q  A  K  N  E  E  T  D  A  W  W  Q  G  L
1921  ACCGCCTGTATAATTCGTTTGTCTACGACTATCTCTATGAAGACCCCAACCACGTGATGGCCTTCGTTGACAATCACGACACCGACCGCTTCCTGGGCAATGGCCGCGACTCGCTGATGC
430   N  R  L  Y  N  S  F  V  Y  D  Y  L  Y  E  D  P  N  H  V  M  A  F  V  D  N  H  D  T  D  R  F  L  G  N  G  R  D  S  L  M
                                                                  Region IV
2041  TGAAACAGGCACTGGCACTGCTGATGACCGTTCGTCGTATTCCTCAGATGTATTATGGTACGGAAATCATGATGAATGGTACGAAGGAGGTGACCGACGGCAACGTGCGCAAGGACTTCC
470   L  K  Q  A  L  A  L  L  M  T  V  R  R  I  P  Q  M  Y  Y  G  T  E  I  M  M  N  G  T  K  E  V  T  D  G  N  V  R  K  D  F
2161  CAGGTGGATTCCCTGGCGACCAGAACAATGCTTTCACCCGCGAGGGACGTACGCAGGCCCAGCAGCAGATGTGGCAGTGGACCAGTCGTCTGCTCCATTGGCGCAAAGGTAACGAGGTGA
510   P  G  G  F  P  G  D  Q  N  N  A  F  T  R  E  G  R  T  Q  A  Q  Q  Q  M  W  Q  W  T  S  R  L  L  H  W  R  K  G  N  E  V
2281  TTACTAAAGGCAAGATGACGCAGTTTATCCCCTTCAACGGCATTTATGTCATTGCCCGTCAATACAATGACCGTACGGCACTCACCATCCTCAACGGCACCACTCGTCCTGCCACGATGG
550   I  T  K  G  K  M  T  Q  F  I  P  F  N  G  I  Y  V  I  A  R  Q  Y  N  D  R  T  A  L  T  I  L  N  G  T  T  R  P  A  T  M
2401  AGGTGAAGCGCTATGCTGAGGTCATCGGGAAGGCCACCCGTGTGAAAGACATCCTCACTGGCCGTTATTACGACCTCTCCACCGACCTCCAGCTCCGTCCCCGCCAGTCGCTGATACTGG
590   E  V  K  R  Y  A  E  V  I  G  K  A  T  R  V  K  D  I  L  T  G  R  Y  Y  D  L  S  T  D  L  Q  L  R  P  R  Q  S  L  I  L
2521  AGTATTGAGAATGGGTAATGATGAATACCTATTTGAACACAGAGAATATTGCCCGCAGCAAGGGTGGGGTAGAGTACCACCCTCTGCGTCCCTTCCTACCTGAGAATGCGAAGGTGCTGT
630   E  Y  *
2641  TCTTAGGCAGCTTTCCGCCACAGCGCAAGCGGTGGTGTATGGACTTCTATTATCCGAATTTCATCAATGACCACTGGCGCATCGAGGGACAGATATTCTTTGGCGACAAGAACCACTTTG
2761  TGGACCTCGAGGCCAAGCGTTTCAAGATTGATGAGATAATTGCGTTCTGCCAAGAAAAAGGCCTCGCGTTCTTCGACACTTCTACGGCTATTCGTCGCCTGCAGGACAACGCCTCCGACA
2881  AATTCCTCGAGGTGGTGGAGCCTACCGACATCCCTGCCCTGCTTTCTCAACTGCCACACCTTCGTGCCATCGTCACCACAGGCGAGAAGGCCACAGAAACCATCTGTGCCTCGCTCGGCA
3001  TCCCAACAATTCCCAAGGTCAACACTTTTGTAACCATTCCCCTCGCCCTTTGGAGAGGGGCCGGGAGTGAGGCTCTTCTCCTCTGGCGCCTCCCTTCCTCCTCCCGCGCCTACCCCTTGT
3121  CCTTCGACAAGAAAGTCGAGGCCTATCGTCAAGTACTCAATTTATTATTATGAAGAAATATTCCATCGCCATTCTCCTCGCCCTCGTCGCACTGACAGGTTGGGCGCAGGTAAATCCCAC
3241  CACCGCCCTGCTCGGTTCGTGGTCGGGTAAATTGCATGTCGGTGCCGCGTCGCTGACGTTGGTACTCCATCTGGAACAAGGCGACGGCTATGTAATAGCATCGTTCGACAGTCCTGACCA
3361  GGGCGCGAAGGGCATTTCCGCTTATAAGGAATTCCTGAGCGACGACTCGCTGGCCTTGAAGGTGGAGCAGATTGGCATGACCTACCGCGCACGGCTCAAGGGCGACACACTCGACGGCAC
3481  CTTTTCGCAAAAGGGTATGTCGCTGCCACTCGTGATGACCAGGGAGTGCCAGAGGTGA
```

Fig. 2. Nucleotide and deduced amino acid sequence of the *amy*A gene. The nucleotide sequence is numbered from the first. The stop codon is indicated by asterisk. The consensus sequences and putative signal peptide are indicated by underline.

(data not show). The calculated pI of AmyA was 5.87.

The characterization of *amy*A clone was identified by sequencing and database search. We extracted genomic DNA from 49 species culturable rumen bacteria in Table 1. The source of *amy*A gene was confirmed by PCR from extracted 49 genomic DNAs using internal primers of *amy*A gene, which shows about 1 kb DNA fragment. No band was confirmed from culturable genomic DNAs. This fact suggests that *amy*A come from unculturable rumen bacteria in cow rumen environment (Table 1).

Table 1 List of culturable rumen bacteria and the results of confirmed PCR using internal primer of *amy*A gene.

Species	Source & Strain	Confirmed PCR[1)]
pCHJ2 (Positive clone)	CRMGL[2)]	+
Acetitomaculum ruminis	ATCC43876	-
Actinobacillus succinogenes	ATCC55618	-
Bifidobacterium adolescentis	DSM20087	-
Bifidobacterium boum	ATCC27917	-
Bifidobacterium merycicicum	ATCC49391	-
Bifidobacterium pseudolongum subsp. *globosum*	ATCC25864	-
Bifidobacterium ruminantium	ATCC49390	-
Bifidobacterium thermophilum	ATCC25866	-
Butyrivibro fibrisolvens	strainOB156C	-
Clostridium aminophilum	Unknown	-
Clostridium cellobioparum	DSM1351	-
Clostridium clostridioforme	strain tB316	-
Clostridium longisporum	ATCC49440	-
Clostridium proteoclasticum	ATCC29084	-
Corynebacterium vitaeruminis	ATCC10234	-
Eubacterium cellulosolvens	strain 2388	-
Eubacterium limosum	ATCC10825	-
Eubacterium oxidoreducens	DSM3217	-
Eubacterium ruminantium	strain GA195	-
Fibrobacter succinogenes	strain S85	-
Lachnobacterium bovis	strain YZ87	-
Lachnospira multipara	strain L14-8	-
Lactobacillus ruminus	ATCC27780	-
Lactobacillus vitulinus	ATCC27783	-
Megasphaera elsdenii	ATCC25940	-
Methanobrevibacter ruminantiu	DSM1093	-
Methanomicrobium mobile	DSM1539	-
Mitsuokella jalaludinii	ATCCBAA-307	-
Oxobacter pfennigii	DSM3222	-
Prauserella rugosa	ATCC43014	-
Prevotella brevis	ATCC19188	-
Prevotella ruminicola subsp. *ruminicola*	ATCC19189	-
Pseudobutyrivibrio ruminis	strain C78	-
Rumincoccus albus	strain Sy3	-
Rumincoccus flavefaciens	strain 007	-
Rumincoccus hansenii	DSM 20285	-
Rumincoccus productus	ATCC27340	-
Schwartzia succinivorans	DSM10502	-
Selenomonas ruminantium subsp. *lactilytica*	strain HD4	-
Selenomonas ruminantium subsp. *ruminantium*	ATCC12561	-
Stenotrophomonas maltophilia	ATCC13637	-
Streptococcus bovis	ATCC33317	-
Succiniclasticum ruminis	DSM9236	-
Succinivibrio dextrinsolvens	strain 24	-
Succinomonas amylolytica	DSM2873	-
Syntrophococcus sucromutans	DSM3224	-
Treponema bryantii	strain B_25	-
Treponema saccharophilum	DSM2985	-
Wolinella succinogenes	ATCC29543	-

[1)] 1.0 kb PCR product with the internal primers, #1762F and #1650R, from *amy*A (pCHJY100).
[2)] CRMGL ; Cow rumen meta-genomic library

Comparison of amino acid sequence of those AmyA from other sources

Nakajima and his colleagues[1,21] identified four short primary sequence motifs, which are present in amylolytic enzyme that have activities. these motifs were present in AmyA and were indicate as regions I to IV in the alignment shown in Table 2. An α-amylase (AmyA) of cow rumen metagenome shared 59.6% amino acid identity with α -amylase (BAD50039) of *Bacteroides fragilis* YCH49, 49.1% with neopullulanase (AAC44970) of *Bacteroides thetaiotaomicron* 5482, 37.0% with cyclomaltodextrinase (CAD32957) of *Flavobacterium* sp. 92, 25.7% with periplasmic α-amylase (BAA07401) of *Xanthomonas campestris* K-11151, 24.1% with cyclomaltodextrinase (CAA44454) of *Bacillus sphaericus* E-244, 23.8% with neopullulanase 2 (Q08751) of *Thermoactinomyces vulgaris*, 23.7% with cyclomaltodextrinase (P29964) of *Thermoanaerobacter ethanolicus*, 23.1% with maltogenic amylase (AAC15072) of *Thermus* sp. IM6501, and 21.8% with cyclomaltodextrinase (BAB18100) of *Thermococcus* sp. B1001.

We constructed a phylogenetic tree of the amylolytic enzyme by the DNAMAN analysis system using above sequences, as shown in Figure 3. The phylogenetic tree showed that the AmyA of cow rumen metagenome was very close to the AmyA of *E. coli*.

Characterization and identification of the AmyA

To characterize the α-amylase in the recombinant *E. coli* cells, direct activity staining technique that allows rapid and specific detection of α-amylase on the polyacrylamide slab gels was employed. The predicted *amy*A gene product

Table 2 Regions conserved among amylolytic enzymes[1)]

Enzyme Source	Amino acid sequences of conserved regions			
	I	II	III	IV
AmyA	DMIFNHSG	GIRMDTYPY	EYPNFNTVGETW	FVDNHDTDR
BAD50039	DMIFNHCG	GIRMDTYPY	EYPNYNTVGETW	FIENHDTDR
AAC44670	DMIFNHCG	GIRQDTHPY	EYPKFNIVGETW	FLDNHDTSR
CAD32957	DVVLSHIG	GLRIDTYGY	EYPRLNMVGEEW	FGGNHDMAR
conserved	*.. * *	**.** ** *	*** * *** *	* *** *

[1)] The underlined amino acids are conserved among all type of amylolytic enzymes, while hose high lighted with an asterisk are conserved among all the enzymes listed here region I, II, III, and IV have been previously defined. AmyA, from cow rumen metagenomic library; BAD50039, *Bacteroides fragilis* YCH46; AAC44670, *Bacteroidesthetaiotaomicron* 5482; CAD32957, *Flavobacterium* sp. 92.

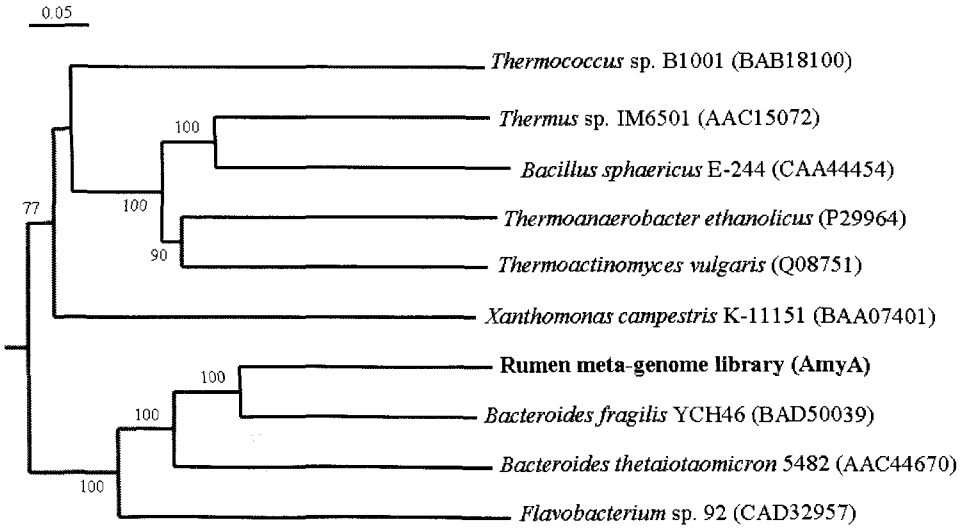

Fig. 3. Phylogenetic tree showing the evolutionary relatedness and levels of homology between the amylolytic enzyme amino acid sequence. The aligned enzymes are from rumen metagenome (AmyA), *Thermococcus* sp. B1001 (BAB18100), *Thermus* sp. IM6501 (AAC15072), *Bacillus sphaericus* E-244 (CAA44454), *Thermoanaerobacter ethanolicus* (P29964), *Thermoactinomyces vulgaris* (Q08751), *Xanthomonas campestris* K-11151 (BAAO7401), *Bacteroides fragilis* YCH49 (BAD50039), *Bacteroides thetaiotaomicron* 5482 (AAC44970), and *Flavobacterium* sp. 92 (CAD32957).

consists of 631 amino acids with an estimated molecular mass of 70,734 Da, which corresponds well with the size of AmyA protein determined by SDS-PAGE (Fig. 4).

The technique takes advantage of the ability of α -amylase to degrade starch even in very small amounts. After electrophoresis, the gel was rinsed, renatured, and incubated. The gel was then stained with staining solution. A white halo appeared around a band where starch was degraded. The pattern was reproducible. A protein band with the apparent molecular weight of about 71 kDa, produced a halo (Fig. 4). The effect of pH on the activity of AmyA against starch was determined at 40℃ in various buffers ranging from pH 3 to 12 (Fig. 5A). Maximal activity was observed at the pH 6.0. The temperature determined by measuring activity at various temperatures in pH 6.0. As with pH dependence, AmyA was also dependent on temperature (Fig. 5B). Maximal activity was observed at 40℃. Divalent cations such as Cu^{2+} and Fe^{2+} in hibited the enzyme activity, while Mg^{2+} (20 mM) and Ca^{2+} (30 mM) enhanced the enzyme activity (Fig. 5C and Fig. 5D).

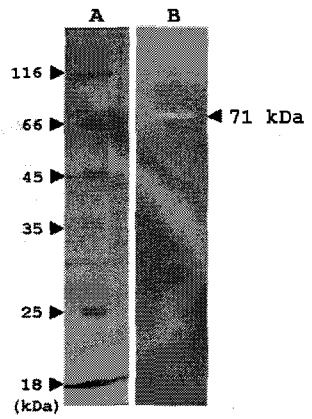

Fig. 4. Detection of amylase enzyme activity band by starch-SDS-PAGE method. Lane A. The molecular weight standard was cut off after electrophoresis and stained with 0.025% Coomassie blue R-250. Molecular weight makers used were β-galactosidase (116,000), bovine serum albumin (66,200), ovalbumin (45,000), lactate dehydrogenase (35,000), restriction endonuclease Bsp 981 (14,400), β-lactoglobulin (18,400), and lysozyme (14,400); Lane B, extract of *E. coli* harboring pCHJY100 expressing AmyA.

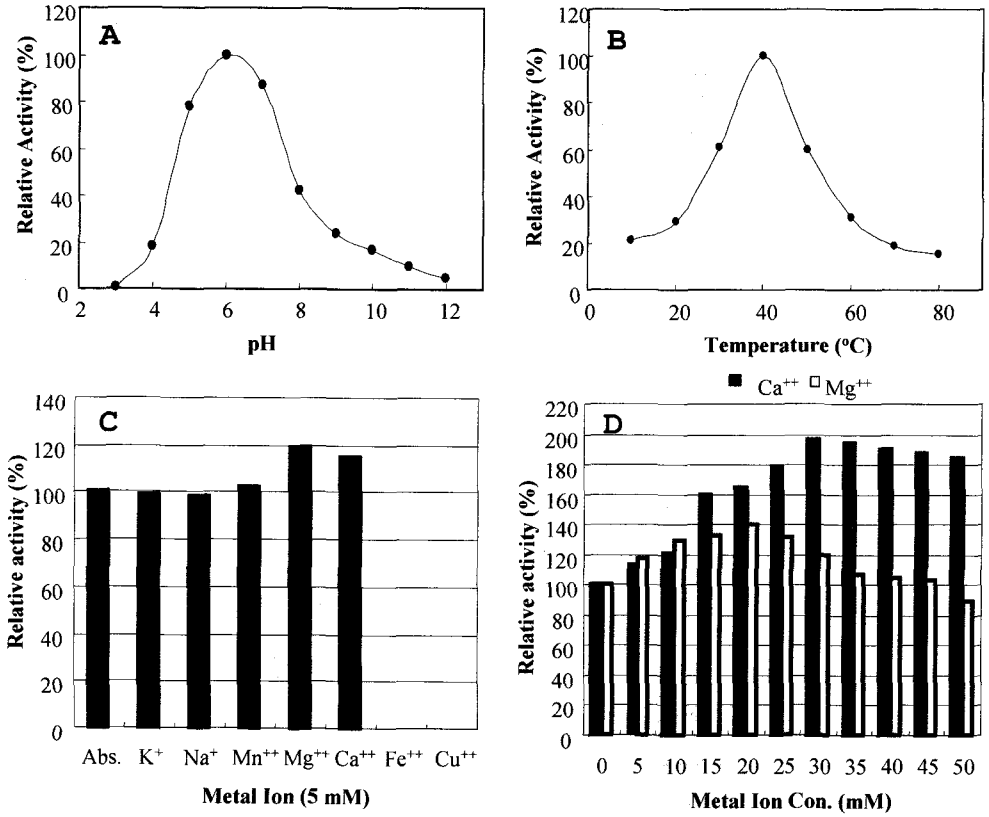

Fig. 5. (A) Effect of pH on the relative activity of AmyA. Enzyme activity was assayed at 40℃ for 30 min at the indicated pH. (B) Effect of temperature on the relative activity of AmyA. Enzyme activity was assayed at pH 6.0 for 30 min at the indicated temperature. (C) Effect of metal ions on the relative activity of AmyA. (D) Effect of calcium and magnesium ions concentration on the relative activity of AmyA.

Discussion

In the study, we utilized a combined precultivation-metagenomics technology for isolation and comparative analysis of amylolytic active gene from a cow rumen. The origin of *amy*A gene could not be confirmed by PCR from extracted genomic DNA of 49 species rumen culturable bacteria. Some researchers only reported the α-amylase gene in rumen bacterial *Streptococcus bovis*[5,6,25,26,35]. In general, cultivation has been successfully used to screen for single microbes or consortia with diverse catabolic capabilities, including the ability to degrade toluene[14] and phthalate[16]. However, only a limited number of reports have described direct cloning from mixed laboratory cultures[11] or enrichments[9] to isolate novel genes or operons useful for biotechnology. Because of the low frequency of finding desirable genes from a metagenomic library of diverse microbial genomes, cloning efficiency is an important factor in constructing a large clone library, which should include most of the microbial DNA[17,19,27]. Here, our library, which was constructed using a rumen total DNA, contained 20,000 clones. Also, an α-amylase from unculturable rumen bacteria sources was screened successfully using cow rumen metagenomic library.

Our results indicate that the amylolytic enzymes obtained in this work could be somewhat different from the amylase of previously cultured rumen bacteria. Examination of amino acid sequences of AmyA from cow rumen metagenomic library, led to the prediction that it would be an extracellular enzyme. It was had typical prokaryotic signal peptide. The amino acid sequences of AmyA are very similar to that of α-amylase (BAD50039) of *Bacteroides fragilis* YCH49, with 59%, identity ot that of neopullulanase (AAC44970) of *Bacteroides thetaiotaomicron* 5482, with 49.1%. The enzymes, which belong to the α-amylase family, share four conserved regions. Regions 1 ot 4, and the catalytic residues of the enzyme are located at region 2, 3, and 4 [1,21]. We could find region 1, 2, 3, and 4 in AmyA. From these results, it was suggested that AmyA of cow rumen metagenome belong to one of the subfamilies of α-amylase .

Acknowledgement

This work was supported by the 21C Frontier Microbial Genopmics and Application Center Program, Ministry of Science and Technology, Republic of Korea.

References

1. Abe, J. I., C. Ushijima and S. Hizukuri. 1999. Expression of the isolamylase gene of *Flavobacterium odoratum* KU in *Escherichia coli* and identification for essential residues of the enzyme by site-directed mutagenesis. *Appl. Environ. Microbiol.* **65**, 4163-4170.
2. Beja, O. M., T. Suzuki, E. V. Koonin, L. A. Hadd, L. P. Nguyen, R. Villacorta, M. Amjadi, C. Garrigues, S. B. Jovanovich, R. A. Fekdman and E. F. DeLong. 2000. Construction and anlysis of bacterial artiticial chomosome libraries from a marine microbial assemblage. *Environ. Microbiol.* **2**, 516-529.
3. Brady, S. F. and J. Clardy. 2000. Long-chain N-acyl amino acid antibiotics isolated from heterologously expressed environmental DNA. *J. Am. Chem. Soc.* **122**, 12903-12904.
4. Brady, S. F., C. J. Chao and J. Clardy. 2002. New natural product families from an environmental DNA (eDNA) gene cluster. *J. Am. Chem. Soc.* **124**, 9968-9969.
5. Brooker, J. D. and J. M. McCarthy. 1996. Gene knockout of the intracellular amylaseg gene by homologous recombinant in *Streptococcus bovis*. *Curr. Microbiol.* **35**, 133-138.
6. Clarke, R. G., Y. J. Hu, M. F. Hynes, R. K. Salmon and K. J. Cheng. 1992. Cloning and expression of an amylase gene from *Streptococcus bovis* in *Escherihia coli*. *Arch. Microbiol.* **157**, 201-204.
7. Courtois, S. C., M. Cappellano, M. Ball, F. X. Francou, P. Normand and G. Helynck. 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. *Appl. Environ. Microbiol.* **69**, 49-55.
8. Dong, G., C. Vieille, A. Savchenko and J. G. Zeikus. 1997. Cloning sequence, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Appl. Environ. Microbiol.* **63**, 3569-3576.
9. Entcheva, P., W. Liebl, A. Johann, T. Hartsch and W. R. Streit. 2001. Direct cloning from enrichment cultures, a reliable strategy for isolation of complete operons and genes from microbial consortia. *Appl. Environ. Microbiol.* **67**, 89-90.
10. Handelsman, J., M. R. Rondon, S. P. Brady, J. Clady and R. M. Goodman. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* **5**, 245-249.
11. Healy, F. G., R. M. Ray, H. C. Aldrich, A. C. Wilkie, L. O. Ingram and K. T. Shanmugam. 1995. Direct isolation of functional genes encoding cellulase from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. *Appl. Microbiol. Biotechnol.* **43**, 667-674.
12. Heme, A. R., R. Daniel, A. Schmitz, G. Gottschaik and R. Daniel. 1999. Construction of environmental DNA libraries in *E. coli* and Screening for the presence of genes conferring utilization of 4-hydroxybutyate. *Appl. Envrion. Microbiol.* **65**, 3901-3907.
13. Henne, A. R., A. Schmitz, M. Bomeke, G. Gottschalk and

R. Daniel. 2000. Screening of environmental DNA libraries for the presence of genes confering lipolytic activity on *E. coli*. *Appl. Envrion. Microbiol.* **66**, 3113-3116.
14. Hubert, C., Y. Shen and G. Voordouw. 1999. Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. *Appl. Environ. Microbiol.* **65**, 3064-3070.
15. Jeang C. L., L. S. Chen, Ming-Y. U. Chen and R. T. Shiau. 2002. Cloning of a gene encoding raw-strarch-digesting amylase from a *Cytophaga* sp. and its expression in *Escherichia coli*. *Appl. Environ. Microbiol.* **68**, 3651-3654.
16. Kleerebezem, R., L. W. H. Pol and G. Lettinga. 1999. Anaerobic degradation of phthalate isomers by methanogenomic consortia. *Appl. Environ. Microbiol.* **65**, 1152-1160.
17. Lee, S. W., K. H. Won, H. K. Lim, J. C. Kim, G. J. Choi and K. Y. Cho. 2004. Screening for novel lipolytic enzymes from uncultured soil microorganisms. *Appl. Microbiol. Biotechnol.* **65**, 720-726.
18. Lim W. J., S. R. Park, C. L. An, J. R. Lee, S. Y. Hong, E. C. Shin, E. J. Kim, J. O. Kim, H. Kim and H. D. Yun. 2003. Cloning and characterization of a thermostable interacellular α-amylase gene from the hyperthermophilic bacterium *Thermotoga maritima* MSB8. *Res. Microbiol.* **154**, 681-687.
19. Lorenz, P., K. Liebeton, F. Niehaus and J. Eck. 2002. Screening for novel enzymes for biocatalytic processes: accessing the metagenome as a resource of novel functional sequence space. *Curr. Opin. Biotechnol.* **13**, 572-577.
20. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Anal. Chem.* **31**, 426-428.
21. Nakajima, R., T. Imanaka and S. Aiba. 1986. Comparison of amino acid sequences of eleven different α-amylase. *Appl. Microbiol. Biotechnol.* **23**, 355-360.
22. Ress Helen, C., S. Grant, B. Jones, D. Grant William and S. Heaph. 2003. Detecting cellulase and esterase enzyme activities encoded by novel genes present in environmental DNA libraries. *Extremophiles* **7**, 415-421.
23. Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman and M. R. Liles. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* **66**, 2541-2547.
24. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratroy Manual*, third eds., Cold Spring Harbor Laboratory PreSS, Cold Spring Harbor, NY, USA.
25. Satoh, E., T. Uchimura, T. Kudo and K. Komagata. 1997. Purification, characterization, and nucieotide sequnce of an intracellular maltotriose-producing α-amylase from *Streptococcus bovis* 148. *Appl. Environ. Microbiol.* **63**, 4941-4944.
26. Satoh, E., Y. Niimura, T. Uchimura, M. Kozaki and K. Komagata. 1993. Molecular cloning and expression of two α-amylase genes from *Streptococcus bovis* 148 in *Escherihia coli*. *Appl. Environ. Microbiol.* **59**, 3669-3673.
27. Schloss, P. D. and J. Handelsman, 2003. Biotechnological prospects from metagenomics. *Curr. Opin. Biotechnol.* **14**, 303-310.
28. Selinger, L. B., C. W. Forsberg and K. J. Cheng. 1996. The rumen: a unique source of enzymes for enhancing livestock production. *Anaerobe* **2**, 263-284.
29. Shin, E. C., B. R. Choi, W. J. Lim, S. Y. Hong, C. L. An, E. J. Kim, K. M. Cho, Y. K. Kim, J. M. An, J. M. Kang, S. S. Lee, H. Kim and H. D. Yun. 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *Anaerobe* **10**, 313-319.
30. Shin, E. C, K. M. Cho, W. J. Lim, S. Y. Hong, C. L. An, E. J. Kim, Y. K. Kim, B. R. Choi, J. M. An, J. M. Kang, H. Kim and H. D. Yun. 2004. Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. *J. Appl. Microbiol.* **97**, 378-383.
31. Shin, E. C., Y. K. Kim, W. J. Lim, S. Y. Hong, C. L. An, K. M. Cho, E. J. Kim, B. R. Choi, J. M. An, J. M. Kang, S. S. Lee, Y. J. Jeong, E. J. Kwon, H. Kim and H. D. Yun. 2004. Phylogenetic analysis of archaea in three fractions of cow rumen based on the 16S rDNA sequence. *J. Agr. Sci.* **142**, 603-611.
32. Shih, N. J. and R. G. Labbe. 1995. Purification and characterization of an extracellular α-amylase from *Clostridium perfringen* type A. *Appl. Environ. Microbiol.* **61**, 1776-1779.
33. Tajima, K., R. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui and Y. Benno. 1999. Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. *FEMS Microbiol. Ecol.* **29**, 159-169.
34. Walker, G. J. 1965. The cell-bound α-amylase of *Streptococcus bovis*. *Biochem. J.* **94**, 289-298.
35. Whitechead, T. R. and M. A. Cotta. 1995. Identification of intracellular amylase activity in *Strepotcoccus bovis* and *Streptococcus salivarius*. *Curr. Microbiol.* **30**, 143-148.
36. Whitford, M. F., R. J. Foster, C. E. Beard, J. Gong and R. M. Teather. 1998. Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. *Anaerobe* **4**, 153-163.

초록 : 소 반추위 메타게놈에서 비배양 세균의 α-amylase 유전자 클로닝

조수정 · 윤한대[1,2]*

(한국생명공학연구원 바이오소재연구부 미생물기능연구실, [1]경상대학교 응용생명과학부, [2]경상대학교 농업생명과학연구원)

미생물 메타게놈은 특이한 생체촉매의 다양한 원료로 제공된다. 한우의 반추위에서 게놈 DNA를 분리한 후 메타게놈 은행을 구축하고 α-amlylase를 암호화하는 유전자를 클로닝하여 DNA 및 아미노산 서열을 밝히고 생화적 특징을 조사하였다. *amy*A 유전자는 1,893 bp로 631개의 아미노산 잔기를 가진 단백질을 암호화였으며 효소의 분자량은 단백질 전기영동 결과 약 71,000 Da으로 확인되었다. 이 효소를 다른 아밀라제와 비교한 결과 21-59%의 상동성을 보였다. AmyA는 pH 6, 40℃에서 최적 활성을 나타내었고, pI값은 5.87이었다. *E. coli* DH5α에서 발현된 AmyA의 활성은 Mg^{2+} (20mM), Ca^{2+} (30 mM) 존재 시 그 활성이 증가하였고, Fe^{2+}, Cu^{2+} 존재 시 저해되었다. *amy*A 유전자의 internal primer를 사용하여 인공적으로 배양할 수 있는 49종의 반추세균에서 분리한 게놈 DNA을 주형으로 PCR 분석한 결과 해당하는 밴드를 확인할 수 없었다. AmyA는 현재로 배양할 수 없는 반추 미생물에서 온 것으로 추정된다.