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Characterization of β-glucosidase produced from *Aspergillus awamori* MIBA335

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Abstract

From the cells and extracellular fluid of *Aspergillus awamori* MIBA335 cultivated on a 20% wheat bran medium and 0.2% carboxymethylcellulose, β-glucosidase (1.5 mg protein) with a specific activity of 88.0 U/mg protein was obtained. The yield was 55.5%, and the enzyme was purified by a factor of 80. The enzyme had a molecular mass of 120 kDa and an isoelectrical point of 4.8. The optimal pH for the enzyme activity for this enzyme was found to be 4.5, and the optimal reaction temperature was found to be 55℃. From the open reading frame (ORF) of the structural genes, which was predicted from the N-terminal amino acid sequence DELAYSPPYY, it was shown that ORF consists of 2935 bases containing 7 exons and 6 introns. There were 860 residues of amino acid translated from 7 exons, and the enzyme was found to be extremely highly homologous (99.9%) with the β-glucosidase AB003470 (gi:2077895) derived from a related species, *A. kawachii*.

Key words: *Aspergillus awamori*, β-glucosidase, genome, *Aspergillus niger*

1. Introduction

The koji mold (*Aspergillus oryzae*) is utilized in the manufacture of products such as liquor, miso and soy sauce, and is found to have high level of safety[1]. This mold is suitable for the mass manufacture of proteins containing various enzymes, and it is especially suited for use as a host in the manufacture of proteins in eukaryotes, which is difficult to achieve with prokaryotes such as *Escherichia coli*[2]. The genome of 37 megabases (Mb) of the *A. oryzae* contains 12,074 genes, and the size of the genome is 7 to 9 Mb larger than those of *Aspergillus nidulans* and *Aspergillus fumigates*. For these reasons, the mold is rich in genes involved in the metabolism of polysaccharides, and is often involved in the synthesis of secondary sugar metabolites[3].

Of cellulase complex enzymes, β-glucosidase (EC 3.2.1.21), also known as β-D-glucoside glucohydrolase, is positioned at the terminal phase and acts as a catalyst in the hydrolysis of the β-glycoside binding in glucose[6-11]. This enzyme is important in reactions involving

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functional aglycons such as rutin, and also in the formation of flavors involving such substances as nerol and geraniol, which provide the characteristic flavors in Shochu spirits[12-14].

In recent years, there is a concern for the depletion of fossil fuels, and there is much expectation for the technology of converting plant biomass into bioethanol. β-glucosidase becomes important in this process, since cellulose needs to be hydrolyzed completely to produce glucose. However, black koji molds involved in the manufacture of Shochu spirits, such as *A. awamori*, were not researched with a view toward application for industrial purposes due to the presence of black pigments in the culture product, and for this reason only a few study results are available for these species of the *Aspergillus* family.

In this study, we have focused on the β-glucosidase in *A. awamori*, and aimed to analyze the enzymatic properties and gene structure of this enzyme.

2. Materials and methods

Cultivation of fungi

The *A. awamori* strain was provided by Akita Konno Co. (Akita, Japan). Passage culture was conducted on this strain using Czapek-Dox minimal agar plates[15], then the strain was stored in the laboratory as *A. awamori* MIBA335.

The spores of MIBA335 were scratched off with a platinum hook and inoculated onto a potato dextrose agar medium[15], then were incubated for 1 week as a static culture at 30°C. The spores obtained were scratched off 3 times with a platinum hook and then suspended in 1 ml of 0.85% NaCl. This spore suspension was used for the following static culture.

Aliquots of colony of *A. awamori* MIBA335 were inoculated to four Erlenmeyer flasks (200 ml), each containing 50 ml of 20% wheat bran and 0.2% carboxymethyl cellulose (CMC) and incubated at 30°C for 4 days. After adding 50 ml of potassium phosphate buffer (pH 6.8) (PPB), the cultures were incubated for 24 hr at 4°C and filtered through a glass filter (pore size, G1). The filtrate was centrifuged at 10,000 rpm for 10 min and at 4°C to remove insoluble materials and finally filled up to 55 ml with PPB. The extracts (200 ml) collected by filtration were kept frozen at -30°C until use.

Purification of enzyme

Step1.

The MIBA335 cell extracts were thawed in 5 ml PPB and homogenized at 4°C with pestle, and then filtered as described above.

Step2.

The homogenate was dialyzed against several changes of 1000 ml of 10 mM PPB and centrifuged. The clear supernatant (350 ml) was applied to DEAE-Sephacel column (2.0 x 60 cm), which was equilibrated with PPB. After washing thoroughly with PPB, the column was eluted with a linear gradient (500 ml) conducted between PPB in a mixing chamber and PPB/1.0M NaCl in a reservoir. Fractions of 15 ml were collected and assayed for enzyme activity. The active enzyme fractions were pooled, dialyzed and concentrated to 5.0 ml on Amicon PM-10 membrane.

Step3.

The concentrate was divided into 2.5 ml each and was applied to Sephacryl S-200 column (2.0 x 100 cm), which was equilibrated with PPB/0.5M NaCl/0.02% sodium azide and eluted with the same buffer. Fractions of 2.0 ml were collected and assayed for enzyme activity. The active enzyme fractions eluted from the column

were collected and concentrated to 2.5 ml as described above. The other portion of the concentrate was applied to the gel filtration chromatography. The two elutes were combined. The final preparation (1.5 mg protein, 88 U/mg protein) was purified 80-fold over the cell extracts (188 mg protein, 1.1 U/mg protein), the yield being 55.5%, and was kept frozen at -30°C until use.

Enzyme assay

β-Glucosidase activity was determined photometrically at 30°C in a reaction mixture (1.0 ml) containing 30 mM PPB, 2 mM *p*-nitrophenyl -β-D-glucopyranoside (β-*p*NPG) and enzyme. One unit (U) of enzyme activity was defined as the amount of enzyme hydrolyzing 1.0 μmol of nitrophenyl glucoside/min under the above conditions[16]. When various sugars were employed as substrates, either reducing power or glucose formed was assayed by using dinitrosalicylic acid and Glucose C test kit (Wako Chemicals), respectively[17]. One unit (U) of enzyme activity was defined as the amount of 1.0 μmol reducing sugars formed/min. Protein was assayed according to the method of Bradford using bovine serum albumin as the standard[18].

Gel electrophoresis

The sodium dodecylsulfate polyacrylamide (SDS-PAGE) was run according to the methods of Laemmli[19]. The SDS gel contained 7.5% acrylamide. In native-PAGE, SDS was deleted from the gel and the running buffer. Electrophoresis was carried out for 90 min at 25 mA/gel and at 25°C. After electrophoresis, the gels were stained with Coomassi brilliant blue for 90 min and decolorized with 25％ethanol/8% acetic acid.

Transblotting of enzyme

The decolorized native gel was placed on top of a PVDF membrane (Transfer Membrane OPTITRAN BA-S85) and then the membrane was blotted by passing through electrical current for 30 minutes at 60 mA[20]. This membrane was then immersed in 10mM β-*p*NPG/PPB and stained for 24 hours at 25°C.

Analysis of N-terminal amino acids

The N-terminal amino acid sequence was analyzed using an amino acid analysis device, Procise 492cLC (Applied Biosystems).

Isoelectric point

Twenty μl of the enzyme reference standard were applied to agarose gel with a pH range of 3.0-8.0 (ATTO). After electrophoresis, the gel was sliced at 2 mm intervals. Each gel section was homogenized in 500 μl of pure water then left standing for 10 minutes. The enzyme activity was measured for 100 μl of the supernatant, and the pH of the remaining supernatant was obtained.

Preparation of genome DNA

After incubation, MIBA 335 fungus bodies were retrieved using a glass filter. These were rinsed with 100 ml of pure water, blotted on filter paper and dehydrated. The genome DNA was retrieved and prepared from the fungus bodies after dehydration using a plant mini kit (QIAGEN) according to the kit protocol.

Primer Design

Primer design was conducted by referencing the base sequence of β-glucosidase AJ132386 (gi:7009580) derived from *A. niger.* The ORF terminal sequence of the related enzyme

AJ132386 was used for forward primer (5'-TTTGCGGCCGCATGAGGTTCACTTTGA TCG-3', containing start codon in the underlined section) and reverse primer (5'-GCGCCTCTAGATTAGTGAACAGT AGGCAGA-3', containing a stop codon in the underlined section).

PCR amplification of glucosidase gene

PCR reaction was conducted for the ORF areas of the β-glucosidase gene, by using the genome DNA as a template, and also using the primer, ORF-F and ORF-R, which correspond to the terminal sequence in the ORF areas. For DNA polymerase, KOD -Plus- (TOYOBO) was used.

In the PCR reaction, thermal denaturation was conducted for 2 min at 94°C. Following this, a series of reactions was performed; annealing was conducted for 15 sec at 94°C, then 30 sec at 58°C and the temperature was maintained at 68°C for 2 min 30 sec as an elongation reaction. This series of reactions was regarded as a cycle, and 30 cycles were performed in total. The resulting substance was stored at 10°C.

The PCR reaction solution was 40 µl in total, comprised of 10X PCR Buffer for -KOD -Plus-, 4 µl; 2 mM dNTPs, 4 µl; 25 mM $MgSO_4$, 1.6 µl; 10 µM ORF-F, 1.2 µl; 10 µM ORF-R, 1.2 µl; KOD -Plus-, 0.8 µl; H2O, 25.2 µl; template DNA (0.68 ng/µl), 2 µl.

3. Results

The properties of the enzyme

Purity and molecular weight

When SDS-PAGE was conducted on the final specimen, a major band was observed near approximately 120 kDa (Fig. 1), and the molecular size matched the results of the Sephacryl S-200 gel filtration chromatography. A minor band (approximately 60 kDa) was also observed, and the results of the investigation with densitometer (600 nm) revealed that the major band corresponds to 95% proteins, and the latter minor band corresponds to 5% protein.

After immersing the native gel in 20 mM β-*p*NPG in 30 mM phosphate buffer (pH 6.5), the band with yellow coloring was extracted. When the N-terminal amino acid sequence was analyzed for this band and the separately excised major band in SDS-PAGE, both bands revealed the N-terminal amino acid sequence DELAYSPPYY, showing a perfect match.

Optimal temperature for activity

When the enzyme was reacted at a pH of 6.8 with the temperature range of 15°C to 80°C, the maximum enzyme activity was observed around 55°C (Fig. 1).

Optimal pH for activity

The enzyme solution and cellobiose (final concentration: 10 mM) were reacted in the buffer with a pH range of 3.0 to 8.0. The results of the measurement on the amount of glucose produced (Fig. 1) showed a peak in glucose level at around pH 4.5.

Thermal stability

The sample was prepared by heating the enzyme solution for 10 minutes so that the temperature of each solution was ranged between 30 and 100°C, and the activity of β-glucosidase was measured. The β-glucosidase activity for the sample maintained at 4°C was regarded as 100%, and the proportion compared to this value was demonstrated in Fig. 1. At 30-50°C, the activity was found to be almost 100%, however at 55°C this was decreased to approximately 60%, and the activity was almost 0% for 60-100°C.

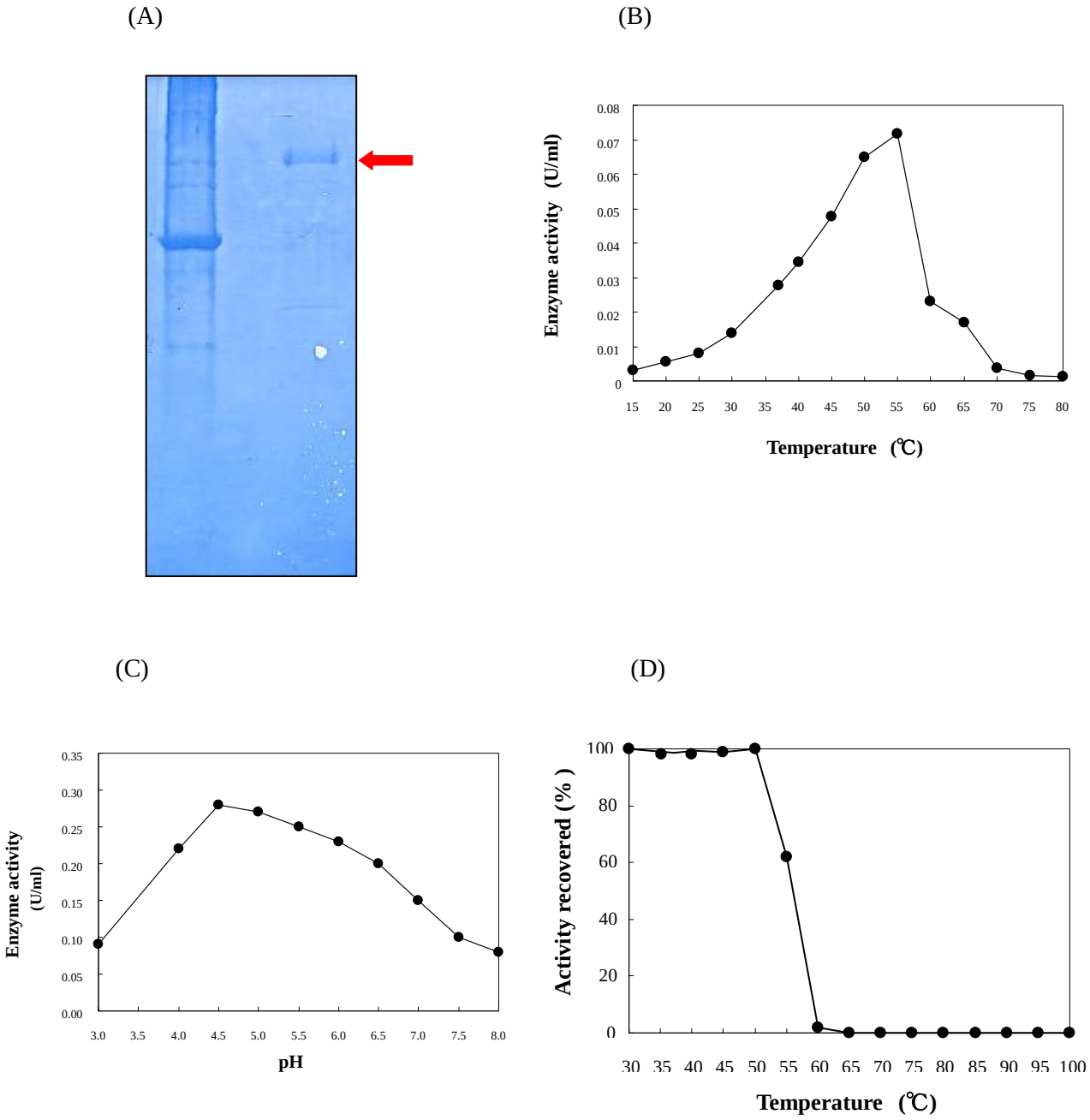

Fig. 1 Properties of *A. awamori* MIBA335 β-glucosidase

(A) SDS gel electrophoresis of the protein. Electrophoresis was carried out for 90 min at 25 mA/gel and at 25°C. Lane M: standard molecular weight markers (222,200, 116,250, 97,400, 66,200, 45,000). Lane S: 5μg MIBA335 β-glucosidase.
(**B) Effect of temperature on the activity of the β-glucosidase.** The activity was determined as given in the text, except that the incubation temperature was changed.
(C) Effect of pH on the activity of rhe β-glucosidase. The activity was determined as given in the text, except that the enzyme was incubated in 10-fold volume each of 100 mM sodium acetate buffer (pH 3.0-5.5), 100 mM Mcllvain buffer (pH 5.0-6.5) and 100 mM potassium phosphate buffer (pH 6.0-8.0), respectively prepared with different pHs, as shown in the figure. The glucose formed was determined with a Glucose C Test kit (Wako Chemical Co.).
(D) Thermostability of the β-glucosidase. The effect of the incubation temperatures on the enzyme was examined after treating the enzyme for 15 min at different temperatures and at pH 6.8, as shown in the figure, and the mixture was assayed for the remaining activities. The activity observed at 4° C was taken as 100%.

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-28                                                    tgctccccctttgggcgaccttgccatc  -1

     exon①
  1  ATG AGG TTC ACT TTG ATT GAG GCG GTG GCT CTC ACT GCT GTC TCG CTG GCC AGC GCT gta   60
  1   M   R   F   T   L   I   E   A   V   A   L   T   A   V   S   L   A   S   A      19

 61  cgtgccgttcctttgtcctgtgaattgcaattgtgctcaattagattcacttgtttgtaccatcatcgctgacaatgg  138

               exon②
139  tctattcatag GAT GAA TTG GCT TAC TCC CCA CCG TAT TAC CCA TCC CCT TGG GCC AAT GGC  200
 20            D   E   L   A   Y   S   P   P   Y   Y   P   S   P   W   A   N   G    36

201  CAG GGC GAC TGG GCG CAG GCA TAC CAG CGC GCT GTT GAT ATT GTC TCG CAG ATG ACA TTG  260
 37   Q   G   D   W   A   Q   A   Y   Q   R   A   V   D   I   V   S   Q   M   T   L   56

261  GCT GAG AAG GTC AAT CTG ACC ACA GGA ACT GG gtaggacttacaaggcgcaatctgtatgctccggc  327
 57   A   E   K   V   N   L   T   T   G   T   G                                    67
                exon③
328  taacaacctctag A TGG GAA TTG GAG CTA TGT GTT GGT CAG ACT GGC GGG GTT CCC CG gt   387
 68                 W   E   L   E   L   C   V   G   Q   T   G   G   V   P   R      82

 exon④
398  aggtttgaaaagaatgtcgagacaggggcattcattgatt aacggcgacag A TTG GGA GTT CCG GGA ATG  458
 83                                                        L   G   V   P   G   M   88

459  TGT TTA CAG GAT AGC CCT CTG GGC GTT CGC GAC T gtaagccatctgctgttgttaggctttgatg   523
 89   C   L   Q   D   S   P   L   G   V   R   D                                    99
   exon⑤
524  ctcttactgacacgtcgcag CC GAC TAC AAC TCT GCT TTC CCT TCC GGT ATG AAC GTG GCT GCA  587
100                      S   D   Y   N   S   A   F   P   S   G   M   N   V   A   A  114

588  ACC TGG GAC AAG AAT CTG GCA TAC CTC CGC GGC AAG GCT ATG GGT CAG GAA TTT AGT GAC  647
115   T   W   D   K   N   L   A   Y   L   R   G   K   A   M   G   Q   E   F   S   D  134

648  AAG GGT GCC GAT ATC CAA TTG GGT CCA GCT GCC GGC CCT CTC GGT AGA AGT CCC GAC GGT  707
135   K   G   A   D   I   Q   L   G   P   A   A   G   P   L   G   R   S   P   D   G  154

708  GGT CGT AAC TGG GAG GGC TTC TCC CCC GAC CCG GCC CTA AGT GGT GTG CTC TTT GCA GAG  767
155   G   R   N   W   E   G   F   S   P   D   P   A   L   S   G   V   L   F   A   E  174
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768  ACC ATC AAG GGT ATC CAA GAT GCT GGT GTG GTC GCG ACG GCT AAG CAC TAC ATT GCC TAC  827
175    T   I   K   G   I   Q   D   A   G   V   V   A   T   A   K   H   Y   I   A   Y  194

828  GAG CAA GAG CAT TTC CGT CAG GCG CCT GAA GCC CAA GGT TAT GGA TTT AAC ATT TCC GAG  887
195    E   Q   E   H   F   R   Q   A   P   E   A   Q   G   Y   G   F [N   I   S] E  214

888  AGT GGA AGC GCG AAC CTC GAC GAT AAG ACT ATG CAC GAG CTG TAC CTC TGG CCC TTC GCG  947
215    S   G   S   A   N   L   D   D   K   T   M   H   E   L   Y   L   W   P   F   A  234

948  GAT GCC ATC CGT GCG GGT GCT GGC GCT GTG ATG TGC TCC TAC AAC CAG ATC AAC AAC AGC 1007
235    D   A   I   R   A   G   A   G   A   V   M   C   S   Y   N   Q   I [N   N   S] 254

1008 TAT GGC TGC CAG AAC AGC TAC ACT CTG AAC AAG CTG CTC AAG GCC GAG CTG GGT TTC CAG 1067
255    Y   G   C   Q   N   S   Y   T   L   N   K   L   L   K   A   E   L   G   F   Q  274

1068 GGC TTT GTC ATG AGT GAT TGG GCG GCT CAC CAT GCT GGT GTG AGT GGT GCT TTG GCA GGA 1127
275    G   F   V   M   S   D   W   A   A   H   H   A   G   V   S   G   A   L   A   G  294

1137 TTG GAT ATG TCT ATG CCA GGA GAC GTC GAC TAC GAC AGT GGT ACG TCT TAC TGG GGT ACA 1187
295    L   D   M   S   M   P   G   D   V   D   Y   D   S   G   T   S   Y   W   G   T  314

1188 AAC CTG ACC GTT AGC GTG CTC AAC GGA ACG GTG CCC CAA TGG CGT GTT GAT GAC ATG GCT 1247
315 [N   L   T] V   S   V   L   N   G   T   V   P   Q   W   R   V   D   D   M   A  334

1248 GTC CGC ATC ATG GCC GCC TAC TAC AAG GTC GGC CGT GAC CGT CTG TGG ACT CCT CCC AAC 1307
335    V   R   I   M   A   A   Y   Y   K   V   G   R   D   R   L   W   T   P   P [N] 345

1308 TTC AGC TCA TGG ACC AGA GAT GAA TAC GGC TAC AAG TAC TAC TAT GTG TCG GAG GGA CCG 1367
355 [F   S] S   W   T   R   D   E   Y   G   Y   K   Y   Y   Y   V   S   E   G   P  374

1368 TAC GAG AAG GTC AAC CAC TAC GTG AAC GTG CAA CGC AAC CAC AGC GAA CTG ATC CGC CGC 1427
375    Y   E   K   V   N   H   Y   V   N   V   Q   R [N   H   S] E   L   I   R   R  394

1428 ATT GGA GCG GAC AGC ACG GTG CTC CTC AAG AAC GAC GGC GCT CTG CCT TTG ACT GGT AAG 1487
395    I   G   A   D   S   T   V   L   L   K   N   D   G   A   L   P   L   T   G   K  414

1488 GAG CGC CTG GTC GCG CTT ATC GGA GAA GAT GCG GGC TCC AAC CCT TAT GGT GCC AAC GGC 1547
415    E   R   L   V   A   L   I   G   E   D   A   G   S   N   P   Y   G   A   N   G  434
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1548  TGC AGT GAC CGT GGA TGC GAC AAT GGA ACA TTG GCG ATG GGC TGG GGA AGT GGT ACT GCC  1607
 435   C   S   D   R   G   C   D  [N   G   T]  L   A   M   G   W   G   S   G   T   A   454

1608  AAC TTC CCA TAC CTG GTG ACC CCC GAG CAG GCC ATC TCA AAC GAG GTG CTC AAG AAC AAG  1667
 455   N   F   P   Y   L   V   T   P   E   Q   A   I   S   N   E   V   L   K   N   K   474

1668  AAT GGT GTA TTC ACC GCC ACC GAT AAC TGG GCT ATC GAT CAG ATT GAG GCG CTT GCT AAG  1727
 475   N   G   V   F   T   A   T   D   N   W   A   I   D   Q   I   E   A   L   A   K   494
 exon⑥
1728  ACC GCC AG  gtaagaagatctccaattcttttgtttcttgtgcaatggatgctgacaacgtgctag T GTC TCT  1799
 495   T   A   S                                                           V   S   499

1800  CTT GTC TTT GTC AAC GCC GAC TCT GGC GAG GGT TAC ATC AAT GTC GAC GGA AAC CTG GGT  1859
 500   L   V   F   V   N   A   D   S   G   E   G   Y   I   N   V   D   G   N   L   G   519

1860  GAC CGC AGG AAC CTG ACC CTG TGG AGG AAC GGC GAT AAT GTG ATC AAG GCT GCT GCT AGC  1919
 520   D   R   R  [N   L   T]  L   W   R   N   G   D   N   V   I   K   A   A   A   S   539

1920  AAC TGC AAC AAC ACC ATT GTT ATC ATT CAC TCT GTC GGC CCA GTC TTG GTT AAC GAA TGG  1979
 540   N   C  [N   N   T]  I   V   I   I   H   S   V   G   P   V   L   V   N   E   W   559

1980  TAC GAC AAC CCC AAT GTT ACC GCT ATT CTC TGG GGT GGT CTG CCC GGT CAG GAG TCT GGC  2039
 560   Y   D   N   P  [N   V   T]  A   I   L   W   G   G   L   P   G   Q   E   S   G   579

2040  AAC TCT CTT GCC GAC GTC CTC TAT GGC CGT GTC AAC CCC GGT GCC AAG TCG CCC TTT ACC  2099
 580   N   S   L   A   D   V   L   Y   G   R   V   N   P   G   A   K   S   P   F   T   599

2100  TGG GGC AAG ACT CGT GAG GCC TAC CAA GAT TAC TTG GTC ACC GAG CCC AAC AAC GGC AAT  2159
 600   W   G   K   T   R   E   A   Y   Q   D   Y   L   V   T   E   P   N   N   G   N   619

2160  GGA GCC CCC CAG GAA GAC TTC GTC GAG GGC GTC TTC ATT GAC TAC CGC GGA TTC GAC AAG  2219
 620   G   A   P   Q   E   D   F   V   E   G   V   F   I   D   Y   R   G   F   D   K   639

2220  CGC AAC GAG ACC CCG ATC TAC GAG TTC GGC TAT GGT CTG AGC TAC ACC ACT TTC AAC TAC  2279
 640   R  [N   E   T]  P   I   Y   E   F   G   Y   G   L   S   Y   T   T   F  [N   Y]  659

2280  TCG AAC CTT GAG GTG CAG GTT CTG AGC GCC CCC GCG TAC GAG CCT GCT TCG GGT GAG ACT  2339
 660  [S]  N   L   E   V   Q   V   L   S   A   P   A   Y   E   P   A   S   G   E   T   679
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2340  GAG GCA GCG CCA ACT TTT GGA GAG GTT GGA AAT GCG TCG AAT TAC CTC TAC CCC GAC GGA  2399
 680   E   A   A   P   T   F   G   E   V   G  [N   A   S]  N   Y   L   Y   P   D   G   699

2400  CTG CAG AAA ATC ACC AAG TTC ATC TAC CCC TGG CTC AAC AGT ACC GAT CTC GAG GCA TCT  2459
 700   L   Q   K   I   T   K   F   I   Y   P   W   L  [N   S   T]  D   L   E   A   S   719

2460  TCT GGG GAT GCT AGC TAC GGA CAG GAC TCC TCG GAC TAT CTT CCC GAG GGA GCC ACC GAT  2519
 720   S   G   D   A   S   Y   G   Q   D   S   S   D   Y   L   P   E   G   A   T   D   739

2520  GGC TCT GCG CAA CCG ATC CTG CCT GCT GGT GGC GGT CCT GGC GGC AAC CCT CGC CTG TAC  2579
 740   G   S   A   Q   P   I   L   P   A   G   G   G   P   G   G   N   P   R   L   Y   759

2580  GAC GAG CTC ATC CGC GTG TCG GTG ACC ATC AAG AAC ACC GGC AAG GTT GCT GGT GAT GAA  2639
 760   D   E   L   I   R   V   S   V   T   I   K   N   T   G   K   V   A   G   D   E   779
                                                                          exon⑦
2640  GTT CCC CAA CTG  gtaagtaacagaagaaccgaacgatgttgaacaaagctaatcagtcgcag TAT GTT TCC  2710
 780   V   P   Q   L                                                      Y   V   S   786

2711  CTT GGC GGC CCC AAC GAG CCC AAG ATC GTG CTG CGT CAA TTC GAG CGC ATC ACG CTG CAG  2770
 787   L   G   G   P   N   E   P   K   I   V   L   R   Q   F   E   R   I   T   L   Q   806

2771  CCG TCA GAG GAG ACG AAG TGG AGC ACG ACT CTG ACG CGC CGT GAC CTT GCA AAC TGG AAT  2830
 807   P   S   E   E   T   K   W   S   T   T   L   T   R   R   D   L   A   N   W   N   826

2831  GTT GAG AAG CAG GAC TGG GAG ATT ACG TCG TAT CCC AAG ATG GTG TTT GTC GGA AGC TCC  2890
 827   V   E   K   Q   D   W   E   I   T   S   Y   P   K   M   V   F   V   G   S   S   846

2891  TCG CGG AAG CTG CCG CTC CGG GCG TCT CTG CCT ACT GTT CAC TAA atagctcttaaatggcata  2954
 847   S   R   K   P   P   L   R   A   S   L   P   T   V   H  stop                    860

2954  ccatgatggccgtggtatatgaattaatgatttatg                                          299
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Fig. 2. Structure of the *A. awamori* β-glucosidase gene.

The first component of the sequence was set as A of ATG, which is the start codon of ORF. The amino acid sequence converted from exon is shown in the lower row of the base sequence. The amino acid shown with square boxes is the predicted additional section of the N-binding sugar chains, and the underlined section corresponds to the N-terminal amino acid sequence. The whole length of ORF consists of 2935 bases, and contains 7 exons and 6 introns.

Isoelectric point

Peak activity was observed in the agarose gel, showing that the corresponding sectional pH was 4.8.

Substrate specificity

Table 1 shows the hydrolytic ability of MIBA335 with various types of sugar. Of the disaccharides, cellobiose is found to act as a substrate, however this did not occur for maltose, which consists of α-1,4-binding. The polysaccharide Avicel, which consists of β-1,4-binding, and CMC did not act as substrates.

Table 1 Hydrolysis of various sugars by *A. awamori* MIBA335 β-glucosidase

Substrate	Concentration	Relative activity (%)*
β-*p*NPG	2 mM	100
α-*p*NPG	2 mM	0
Cellobiose	2 mM	30.3
Lactose	2 mM	0
Maltose	2 mM	0
Avicel	0.1 %	0
CMC	0.1 %	0

*The enzyme activity in the standard measurement condition was taken as 100%. α-pNPG refers to *p*-nitrophenyl-α-1,4- gluco -pyranoside.

Base sequence of the *A. awamori* MIBA 335 gene

The whole length of the base sequence, excluding the primer sequence, was 3019 bases (Fig. 2). Using the determined 3019 bases as a query, a homology search was conducted with NCBI blast, mainly for enzymes which belong to family 3[26, 27]. *A. kawachii* β-glucosidase AB00 3470 (gi:2077895), which has the same 10 residues in the N-terminal amino acid sequence, was found to have the highest level of homology at 99.9%, and 3017 of 3021 bases in AB003470 matched with the query[20]. The next highest level of homology was observed in β-glucosidase AJ132386 (gi:7009580) derived from *A. niger*, with 96.7% homology, with 2921 of 3020 bases in AJ132386 matching with the query.

The ORF sequence was found to consist of 2935 bases, with 7 exons and 6 introns (Fig. 7). There are 860 residues of amino acids translated from the 7 exons. These results matched those of β-glucosidase AB003470 (gi:2077895) derived from *A. kawachii*, which was found to have the highest level of homology, as well as β-glucosidase AJ132386 (gi:7009580) derived from *A. niger,* which was found to have the second highest level of homology.

The β-glucosidase derived from *A. kawachi* and *A. niger* both consist of 860 residues of amino acids. The same sequence as the MIBA 335 enzyme gene can be found from 20 to 29 residues counting from the N-terminus, however the amino acid residues 1-19, on the upstream, are found to be signal peptides according to the literature[28, 29]. Therefore, it was found that the 20 residues of amino acids are N-terminal amino acids, and the enzyme being tested has the identical amino acid sequences in the N-terminal region as AJ132386 and AB003470.

4. Discussion

Not much is known in regards to the taxonomy of *A. kawachii*, *A. niger* and *A. awamori*.

The white koji mold *A. kawachii* is thought to have derived from the black koji mold by mutation, however some researchers regard *A. kawachii* and *A. awamori* as the same strain[28]. The DNA and deduced amino acid sequence of *A. kawachii var. awamori* are 91.5% and 95.8%, respectively identical to *A. niger*.[29]

The comparison of the base sequence of MIBA335 β-glucosidase with that of *A. kawachii* β-glucosidase revealed two differences. One is that there is a missing base in the 2nd intron (i2). In *A. kawachii*, c was inserted in the 309th base, however this was deficient in *A. awamori*. However, since this deficiency appears at the intron section, this is not reflected in the amino acid sequence. The other difference was found in 1 base of the 6th exon (E6). In *A. awamori* the 1867th base is G, however this is A in *A. kawachii*. This resulted in a 1 amino acid difference between *A. kawachii* and *A. awamori*.

In MIBA335, the codon AGG appears from the 1866th base, which becomes arginine when translated. However, in *A. kawachii* the codon is AAG, which translates into lysine. However, since both arginine and lysine are basic amino acids, it is thought to have almost no effect on the 3D structure of the proteins.

Since AB003470, derived from *A. kawachii*, belongs to family 3, there is a need to check the homology of the base sequence with the enzymes from the same family[24-27], as well as the presence of conserved domain[30-34]. The condition for family 3 includes the presence of conserved domains at 2 locations, the N-terminal side and C-terminal side. The conserved domain on the N-terminal side must have an amino acid sequence with a barrel structure consisting of 8 each of the α-helix structure and β-sheet structure, with glutamic acid, important for the hydrolytic activity, contained in the central section. The conserved domain on the C-terminal side must have an amino acid sequence with a sheet structure consisting of 6 each of the α-helix structure and β-sheet structure, with glutamic acid, important for the hydrolytic activity, contained in the central section[24].

When the sequence of MIBA335 β-glucosidase was compared with the data in the NCBI conserved domain, the conserved domain on the N-terminal side was observed in 77-300 residues, and aspartic acid, important for activities, were observed in 280 residues. Conserved domains on the C-terminal side were observed in 400-644 residues, and glutamic acid, important for activities, was observed in 490 residues. It is estimated that β-glucosidase is a family 3 glucohydrolase with a 2935-base sequence including 7 exons and 6 introns, with 860 residues of amino acids.

The strains *A. awamori*, *A. kawachi* and *A. niger* were defined as being closely related from morphological studies, but they may be unified into a single species in the future with the advancement in molecular biological classification using phylogenetic analysis[36]. There is a possibility that new uses for *A. awamori*, which was not used in the food processing industry due to the presence of black pigments, may be found for pharmaceutical areas[37].

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