Sequence Analysis and Heterologous Expression of Rhamnogalacturonan Lyase A Gene (*AsrglA*) from *Shoyu Koji* Mold, *Aspergillus sojae* KBN1340

Shoko YOSHINO-YASUDA^{1*}, Shuichi KARITA², Masashi KATO³ and Noriyuki KITAMOTO¹

¹ Food Research Center, Aichi Center for Industry and Science Technology, 2-1-1 Shimpukuji-cho, Nishi-ku, Nagoya, Aichi 451-0083, Japan

² Graduate School of Regional Innovation Studies, Mie University, 1577 Kurimamachiya-cho, Tsu, Mie 514-8507, Japan

³ Department of Applied Biological Chemistry, Faculty of Agriculture, Meijo University, 1-501 Shiogamaguchi, Tempaku-ku, Nagoya, Aichi 468-8502, Japan

Received July 6, 2012; Accepted August 20, 2012

The rhamnogalacturonan lyase A gene, designated *AsrglA*, was isolated from a *shoyu koji* mold, *Aspergillus sojae* KBN1340, and characterized. The structural gene comprised 2,245 bp with 11 introns. The open reading frame encoded a 528-residue protein with a signal peptide of 20 residues. *AsrglA* shared a high degree of sequence identity with *Aspergillus oryzae* rhamnogalacturonate lyase A and *Aspergillus flavus* rhamnogalacturonase B. Utilizing the promoter of the *A. oryzae* *taaG2* gene, AsRglA was successfully expressed in *A. oryzae* and secreted into the culture medium. AsRglA had a molecular mass of 55.0 kDa, a pH optimum of 4.5, and a temperature optimum of 50°C.

Keywords: *shoyu koji* mold, *Aspergillus sojae*, rhamnogalacturonan lyase gene, heterologous expression

Introduction

The filamentous fungus *Aspergillus sojae* is used for the production of fermented soy sauce (*shoyu*), a traditional Japanese food. *A. sojae*, when grown on a *shoyu koji* composed of soybean and wheat, secretes a large variety of carbohydrases and proteases. These enzymes are essential for the efficient maceration and hydrolysis of soybean and wheat. Among these enzymes, pectinolytic enzymes are the most important in soy sauce production as they improve the filtration efficiency of soy sauce mash and decrease the amount of the pressed cake (Kikuchi, 1977). Pectinolytic enzymes play a key role in the complete degradation of the plant cell wall, as the depolymerization of pectin exposes other cell wall components to degradation and leads to further cell wall breakdown. Pectin is one of the most complex polysaccharides with a backbone composed of homogalacturonan “smooth” regions and highly rhamnified “hairy” regions of rhamnogalacturonan (Willats *et al.*, 2001). The canonical pectinolytic enzymes such as polygalacturonases and pectin lyases can fragment homogalacturonan but cannot degrade rhamnogalacturonan. Breakdown of rhamnogalacturonan is

mainly mediated by two rhamnogalacturonan-degrading enzymes, rhamnogalacturonan hydrolase (RGaseA) and rhamnogalacturonan lyase (RGaseB). The rhamnogalacturonan-degrading system of *Aspergillus aculeatus* has been well characterized, and three-dimensional structures of RGaseA and RGaseB have been elucidated, contributing toward the understanding of their structure-function relationship (Kofod *et al.*, 1994, Mutter *et al.*, 1994, Mutter *et al.*, 1996a, Mutter *et al.*, 1996b, Mutter *et al.*, 1998, McDonough *et al.*, 2004, Jensen *et al.*, 2010). Furthermore, the usefulness of these enzymes in the processing of food has been confirmed. The solubilization of soybean cell wall material (CWM) by RGaseA and RGaseB in combination with other pectinolytic enzymes was compared in order to identify enzymes for new soybean processes or products (Heldt-Hansen *et al.*, 1996). RGaseA was only able to solubilize 7% of the CWM, whereas addition of rhamnogalacturonan acetyl esterase and galactanase increased the solubilization to 17% and 44%, respectively. On the other hand, the solubilization of the CWM by RGaseB was less dependent on the presence of the other pectinolytic enzymes, as 37% are solubilized with RGaseB alone. These results show that the ability of RGaseB to solubilize soybean fibers may be useful for improving the filtration efficiency of soy sauce mash and decreasing the amount

*To whom correspondence should be addressed.

E-mail: shiyouko_yasuda@pref.aichi.lg.jp

of pressed cake.

Here, we describe the cloning and sequencing of a rhamnogalacturonan lyase gene, the *AsrglA* gene, from an industrial *shoyu koji* mold strain, *A. sojae* KBN1340. In addition, the *AsrglA* gene was overexpressed in *Aspergillus oryzae* under control of the promoter of the *A. oryzae* *taaG2* gene (Tsukagoshi *et al.*, 1989). The recombinant *AsrglA* protein was purified and its enzymatic properties were characterized.

Materials and Methods

Fungal strain, culture media and transformation An industrial *shoyu koji* mold, *A. sojae* KBN1340, obtained from Bio'c (Toyohashi, Aichi, Japan) was used for DNA isolation. The *pyrG*-deficient strain, *A. oryzae* KBN616-P3, derived from an industrial *shoyu koji* mold, *A. oryzae* KBN616, was used for transformation. Rice bran (RB) medium containing 3% rice bran, 1% polypeptone, 0.5% KH₂PO₄, 0.5% KCl, 0.1% NaNO₃, and 0.05% MgSO₄·7H₂O or starch polypeptone (SP) medium containing soluble starch instead of rice starch in RB medium were used for liquid cultivation of *A. oryzae*. AGM agar medium containing 0.3% NaNO₃, 0.1% KH₂PO₄, 0.05% KCl, 0.005% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.1% AZCL-galactan (MegaZyme, Australia), 2.0% maltose, and 1.5% agar was used for selection of the high AsRglA-producing *A. oryzae* transformant. Protoplast transformation of *A. oryzae* was carried out as described previously (Kitamoto *et al.*, 1995).

Amplification of *AsrglA* gene fragments by PCR A genomic DNA fragment encoding a portion of *AsrglA* gene was amplified by PCR using genomic DNA from *A. sojae* KBN1340 as a template. Two oligonucleotide primers were synthesized based on two amino acid sequences conserved among *A. aculeatus* RhgB (Kofod *et al.*, 1994), *Aspergillus niger* RglA (Pel *et al.*, 2007) and *Neurospora crassa* rhamnogalacturonase (Nelson *et al.*, 1997). The sense primer rhgB1 (5'-CAYATGGCYACYTAYATYAC-3') was homologous to the sense strand for amino acid residues 111 to 117 of the *A. aculeatus* RhgB. The antisense primer rhgB4 (5'-TCCCCARTCRCCRATYTTRAA-3') was complementary to the sense strand for amino acid residues 362 to 368 of *A. aculeatus* RhgB. The amplified 1,090-bp fragment designated RHG1 was cloned into pUC118 and sequenced. For amplification of the 5'- and 3'-regions of RHG1, cassette-ligation-mediated PCR (CLM-PCR) was performed using LA PCR *in vitro* Cloning kit (Takara Bio, Otsu, Shiga, Japan) as described by Isegawa *et al.* (Isegawa *et al.* 1992). Four specific primers, rhgB5 (5'-TGTACTTACCACATCCGAGCCTTCG-3'), rhgB6 (5'-ATGTCTCTGGTCATCGATGAAACGC-3'), rhgB7 (5'-GAACTGTATCCGGAAGTGCATCTGG-3') and rhgB8

(5'-GGACATACACATCTTCAGACGGTAG-3'), were synthesized based on RHG1. The 5'-region was amplified from a *Sa*II cassette-ligated genomic DNA with two sets of primers (C1/rhgB6 in the primary amplification and C2/rhgB5 in the second round of PCR) (Fig. 1). The 3'-region was also amplified from an *Eco*RI cassette-ligated genomic DNA with two sets of primers (C1/rhgB7 in the primary amplification and C2/rhgB8 in the second round of PCR) with a similar procedure. The amplified fragments, the 718-bp fragment for 5'-region and the 3,461-bp fragment for 3'-region, were cloned and sequenced.

Construction of expression vector for *AsrglA* gene An expression vector, pTARB100, for the *AsrglA* gene under the control of the *A. oryzae* *taaG2* gene promoter was constructed as follows. The 618-bp fragment of the *A. oryzae* *taaG2* gene promoter was amplified by PCR using the *A. oryzae* *taaG2* gene cloned into pUC19-TAA (Tsukagoshi *et al.*, 1989) as a template, and a pair of primers, TAAP2 (5'-GTGAATTCATGGTGTGTTTGTATCATTT-3') corresponding to positions -615 to -590, and TAAPRO (5'-CCGAGCTCATGCATAAATGCCTTCTGTGG-3'), corresponding to positions -15 to +3, with the translation start site of the *A. oryzae* *taaG2* gene being +1. The *Eco*RI and *Sac*I digested PCR-amplified DNA fragment was inserted into the *Eco*RI-*Sac*I site of pYRM100 (Yoshino-Yasuda *et al.*, 2011), resulting in plasmid pYRMTA100. The 1,359-bp fragment encoding the signal peptide and the mature protein of the *AsrglA* gene was amplified by PCR using chromosomal DNA from *A. sojae* KBN1340 as a template, and a pair of primers, rhgBN3 (5'-AAATGCATTCCAGAACCATTCTTTTCTCAACT-3'), corresponding to positions +7 to +30, and rhgBC (5'-CTACTAGTACTTTACGCCTGGTCAG-3'), corresponding to positions +3,108 to +3,132, with the translation start site of the *AsrglA* gene being +1. The *Eco*T22I- and *Spe*I-digested PCR-amplified DNA fragment was inserted into the *Eco*T22I-*Xba*I site on pYRMTA100. The resultant plasmid, pTARB100, was introduced into *A. oryzae* KBN616-P3.

Sequence analysis of *AsrglA* cDNA fragment A cDNA fragment of the *AsrglA* gene was amplified by RT-PCR using a High-fidelity RNA PCR Kit (Takara Bio) as follows. Total RNA was isolated from the high AsRglA-producing *A. oryzae* transformant cultured in SP medium. From total RNA, first-strand cDNA was synthesized using Oligo dT-Adaptor primer FB (Takara Bio) and was subjected to PCR using a pair of primers; rhgBN3 and Adaptor primer FB (Takara Bio). A fragment of approximately 1,700 bp was amplified and cloned into pUC119.

Purification and amino acid sequencing of *AsrglA* from overexpressing *A. oryzae* transformant High AsRglA-producing *A. oryzae* transformant SS28 was grown in RB me-

Fig. 1. Amplification scheme of the 5'-flanking region of RHG1. The 5'-flanking regions of RHG1 and RHG2 were amplified as described in the Materials and Methods. Black, gray and white boxes indicate the *SalI* Cassette, RHG1 and the flanking regions of RHG1, respectively. Arrows indicate the position of oligonucleotide primers used.

dium at 30°C for 4 days. After removal of mycelia through filtration, 25 mL of the culture filtrate was dialyzed against 10 mM Tris-HCl buffer (pH 7.0). Dialysate was loaded onto a HR16/20 Fast Flow Q-Sepharose anion-exchange column (GE Healthcare, Buckinghamshire, UK) equilibrated in the same buffer. Adsorbed proteins were then eluted with a linear gradient from 0.25 M to 0.4 M NaCl.

In order to analyze the N-terminal amino acid sequence, purified enzyme was blotted onto a PVDF membrane (Bio-Rad Laboratories, CA, USA) and sequenced on an Applied Biosystems 491cLC sequencer (Applied Biosystems, CA, USA).

Enzyme assay Rhamnogalacturonan lyase activity was determined by the slightly modified method described by Kofod *et al.* (Kofod *et al.*, 1994). One milliliter of 0.2% suspensions of AZCL-galactan and 50 μ L of enzyme solution were incubated at 40°C for 15 min in 50 mM sodium acetate buffer (pH 5.0) with shaking, followed by enzyme inactivation at 95°C for 20 min. After centrifugation, the release of blue color into the supernatant was measured at 595 nm. All assays were performed in triplicate, and appropriate blanks

were subtracted. One arbitrary enzyme unit was defined as an increase of 0.01 in absorbance per min per mL of enzyme solution. The pH optimum was determined in 50 mM sodium acetate buffer at various pH levels (3.0 to 7.0) and the temperature optimum was determined at various temperatures (30°C to 65°C) in 50 mM sodium acetate buffer (pH 5.0). The thermal and pH stabilities were determined by incubating the enzyme at various temperatures (30°C to 65°C) for 30 min and at various pH levels (3.0 to 7.0) for 17 h at 4°C, respectively.

Results and Discussion

Structural features of the *AsrglA* gene Part of the *AsrglA* gene was amplified by PCR with the genomic DNA as a template and a pair of degenerated primers corresponding to the amino acid sequences in the regions conserved among rhamnogalacturonan lyases from *A. aculeatus*, *A. niger* and *N. crassa*. The amplified 1,090-bp fragment designated as RHG1 was sequenced and found to contain an open reading frame encoding 258 amino acids bearing high homology to the sequences of the rhamnogalacturonan lyases from *A.*

CTCGAGACTAGTAGAACTCATGTGGGAGCGAA <u>TATAAAGAGACAATGGCCGACAATATGACGAATATTGGGAAGGGGATACTACTCTGCCGAGATTGACAGACAAAATGTTGTCCAGA</u>	12
	M L S R 4
ACCATTCTTTTCTCAACTTCTTTTCTTTGGGCCAGAGTCGCCAATGCGGCCTTTGGAATCACCACCTCTGACGACTCTACGTTATTGACGCAGGGTCGGCAAAACCCTCTAAATTTTACG	132
T I L F S T S F L W A R V A N A <u>A F G I T T S D D S</u> Y V I D A G S A N P L K F T	44
GTTTCCCGTTCGAGTCGCATATCACCTCTATCAATTACTATGGCTCAGAACTTCAGTACTCCGGCAGCGGCAGCCATCGGTTCTGGCCTTGAAGCGCAGATGTTTCTCGCGTAGAA	252
V S R S S C D I T S I N Y Y G S E L Q Y S G T G S H I G S G L G S A D V S A V E	84
GATGgtgagctaacgatacaacaagctatattgaccatgtatatactgacaccatgtctgcaaagGGGACTACATCAAGGTTACCTGCGACACCGATACCTTGACACAGTATTTCGTTGT	372
D G D Y I K V T C D T D T L T Q Y F V V	104
CCACAATGGCGACTCAGTCATTACATGGCGACGTACACCACAGAGGgttagttcacgtcacggcgcaagaagaatttcctgacattcgtcttaataatccagACCTCCGTTGG	492
H N G D S V I H M A T Y T T E E P S V G	124
TGAGTTGCGGTTTCATTGCACGCCTTAACCTCGGAGCTCTACCGAACGAGGAACCGTTTCGGCGATGTGTCCACCCTTCGGGCGGCGAGGCAATCGAAGGCTCGGATGTGtaagtacaaa	612
E L R F I A R L N S E L L P N E E P F G D V S T T S G G E A I E G S D V	160
aatgaagctagggcacgaaggttagctgatgaagctatatatagTACCTTGATAGTGGGAGACCCGTAGCAAGTTCTACTCCAGCCAGCGTTTCATCGATGACCGAGACATTGtaagg	732
Y L V D G E T R S K F Y S S Q R F I D D Q R H C	184
acgtacttgcgtgactgtagtgcatggctaacctgcttctagGTGTGCTGGAGACGCCACCGTGTTCGATGATCTCAACCAATACGAGACTTCTCTGGTGGTCTTTCTCCGgt	852
V A G D A H R V C M I L N Q Y E T S S G G P F F R	209
cagtacactactgtgacgtaacatgcttacatactaattttaaagCGATATCAACTCCAACACGGCGGTAGTTATAACTCCCTCTACTGGTACATGgtacgtttgacacgacatta	972
D I N S N N G G S Y N S L Y W Y M	226
gcttttcaattattttgttaacgaagcgagAACTCGGGCCATGTCCAACAGAGAAGACAGCGCAGGGTCTCCATGGGCGATACTCTATGTACTTCAGCCGAGTGGAACACCCAGCAC	1092
N S G H V Q T E D R R Q G L H G P Y S M Y F S R S G T P S T	256
CGATATGACACATCTTCTTCGCAACCTGGACATCAAGGGCTACGTGCTGACGCGCGCGGAATGTATCCGGAATGCATCTGGAGCCGACTCCAGCTTTAAATGGGTCGTTC	1212
D I D T S F F A N L D I K G Y V A A D G R G T V S G T A S G A D S S F K W V V H	296
CTGgtacgataccgtgactaacagaatctaaatctgacctaacacaataacagGTACAATGCCGATGCCAGTACTGGACATACACATCTTCAGACGGTAGCTTCACTTCCCGGCC	1332
W Y N A D A Q Y W T Y T S S D G S F T S P A	318
ATGAAGCCAGGTGATTACACCATGGTCTACTACCAAGGCGAATACAAGGTAGCCGAGACCTCTGTATCGGTTACAGCCGATCATCAACAGCAAGGACATCTCTGGATCCGTGGAAACA	1452
M K P G D Y T M V Y Y Q G E Y K V A E T S V S V T A G S S T S K D I S G S V E T	358
GGCGATACCATTTTCAAGATCGCGCAGTGGGACGGAACgtacgtgtcctattccaacccctgctagctagcaagccaccacatgagcataactgaattatcatctctagACCAACCGGC	1572
G D T I F K I G D W D G T P T G	374
TTCCGCAACGCAGAGAACCAACTGCGGATGCACCCCTCGGACTCACGCATGTCTCTTTGGGACCCCTTACCTACACTGTAGGAAGCTCCGAGCTCACCGACTTCCCAATGGCTGCCTTC	1692
F R N A E N Q L R M H P S D S R M S S W G P L T Y T V G S S E L T D F P M A A F	414
AAGGGAGTTAAAGACCCCGTACCAATTAATTCACGGCCACTTCCGCTCAGACCGCGCGCTACTCTGCGCATCGGAACGACCTCTCGTTGCGTGGCGGTGCTCCTCAAGCTACTGta	1812
K G V N D P V T I K F T A T S A Q T G A A T L R I G T T L S F A G G R P Q A T	453
gaaacccagtgcaaaaccgactcacatccccatagactgggaacaaaatagaactaacaacaaaccagATCAACGACTACGAAGGCTCCGCCCCCTGTCAGCCCAACAACTTCGACTCC	1932
I N D Y E G S A P S A P T N L D S	470
CGCGGCGTCACCCGCGGAGCTACCGCGGCCCTCGGCGAAGTCTACGACGTGAGCATTCATCTGGAACAATCGTCGAAGGAGAGAACACTgtgcgcatacctcaagatccttctccccct	2052
R G V T R G A Y R G L G E V Y D V S I P S G T I V E G E N T	500
aatcaaaagttcataattaacacaacctcagATCAATCAGCGTCATCTCTGGAAGTCCGGCGATGAGTTCCTTGTCTCTAATTTGtaagtgcttactatttttcattgggttatt	2172
I T I S V I S G S S G T D E F L A P N F	519
GtcttggtgtggttaattggctaattgaattactgttctagATCTTTGACTCGTCGAAGTGTTCAGTAAGGATGTGAATAGTGTATCCAGTGGAAATTGTACATATTGATTGATGCT	2292
I F D C V E L F Q *	528
GGCTATGAAATGACCAGCGTTTGTGTAGTAGTAAGGTTCAACGGGTCTATCTTCATTAATTGAATTATTTTGTACTTTGAGCTGGGCATTGT <u>ATAAG</u> GATTGTTAAACGGACACCCCA	2412
TCTGACTATATCAACACCATACTCTGCCCTCCGAAACAAAAAGAACTAGATTGACTCAGTACTTGCGAATGCCCTAAAGGCTACAAAAATCTCATATAGTATCCCAACACAATTCTC	2532
ACCTATTCCTATCAAGGTAAGGCACATCTTCCACAACAACCTACCTACCCCTAACACAATCATACCATAGCTACATGGACATAGACCCACTCCAATATAAGCAATTCCCTAGTCTATTACC	2652
CCCTAAGATCACCTATCTCACCACAACCCCTTCAAGATAATCAAACTCTGAAATCCATCTCAATCCCTCAATCAGACCCCAAGACCAATCCCAAGATCGTCAATCCAAAGACTACG	2772
GTCTCATGGAAGACCGAAACGAGAATAGCTCGGCACAGGGCTTTGGGAAGGTAATTTCAAGTTTACCAGAGGGTAGAGCGGGAGCTTGTCTCCGAGAAGCGGCGCTACAAGGCTGAAC	2892
TTTGGTTCAAGTGAGTTCCGGAGGAGGACTAGTTTACCTAGCGTGGAGATTGTAGTTTATTTCTGAAATCTTTTGTGTTTTTTTAGACAGCTATTTGATACGCGTCGTATTCA	3012
GTTAGTACATTTGCTTGTGATTTTAGGTGTACGCTATCTGGAGTATATAGATGATTGTATCGACCACGCGGTGTTTCTAAAGCCATATTTCTCTGACACGCGTAAAGTACTAGTAG	3232
TAAGCGGTTAATTTACAAGCCGTACAAAAGGTTGTGCGTGGAGATATAGCTGTGAATAGCCTCGAAGTGGGTTTTAGTGTATCATTTTCCACAGGTGCTACCCCTCTTATAAAGT	3352
TATATCTTGTGAAATTATAAGGTTAATTATCTGTATGATCATCAGGTGAAATCCGAGATGGTCAAAAACCATCTATAAGATAAAGAGAACAGTCTATATCTTACAACCTCAACCT	3472
CCTAAGAGCAACAACAGCACCAACGCCACAACACCGGCGCTCAAAACCAAGTCCACCAACAGACCGAATCCACCCCTCAACAGCTTCTCCCATGCACAGCTTTCTCCCAACCA	3592
GACACCCCTTAACGTTACGATCATTACCACTCCACACCACTTTGTGGATCTTCTTCTCCACGCTCTCCCATCAAAAGACATCAACCTGGCTTTCACCATTTGTATACCGGCTCGCAGGTA	3712
AAACGCCCAAAATGGATCAGCTGCGCATCGAACAGCATGCCGTCTGCAATCCACCGATATCATTTCTCCATATTGACCGCAGCTGCGCCACCACTGTAGCCAGGTACAGAGCCTCTTC	3832
CACGCTCAGGACGAGTTTCTTTCCTTCATCTCTCTGCTGGCGGTTCCACATGCCGAAGTAACCGGATACGAGACACGCTTGTGCGGACGCTTCAAGATACTGGGCTATATCCGCC	3952
GCTAACATCCGTCGCGAGACGACAGTGATTCCTTGTGAGGAGCTTGCAGACGGGACAGATGCCAGACCTAAGCGAGATTGACGCGGACAGTGAGAGACCTTGGCGCTCGGTC	4072
GTGGATAAGATCTTGTCTGTCGGCGATAGATGGACGCCGTGGGCGAGGATAGTTCTGTTGTAGAGGCGGTATGTGTCGTACAGCTCGCGTAGCTCTTGGCACTTGGGAATTGGCA	4192
GTGGACGTCGTGGATTTCTTGTGTGTTCTCGGATATGTGCGTTTGGATGTGCGATGGGGGTTTCGTAAGAAGAGGCTAGGTGCGGTAGACCACGATTGCTTTCGGGGTGCATGTTAATGC	4312
AAAGCGGGGTAATTATCGGCTTTATCATGGTTCTTTCGGATCAATGGATGTAGTACGAGATGACTTCTTGTGTTAGCTCGACGAGTCTTTCGGTTGAGGGTTCGATATAGTAGG	4432
CTCAGAGAAGTTTCGGATTGTCCATGCAACACGCGCCGATGAGGCGCGCTTGGCGAGAGCTGCCAGCAAAATTCGTAGCCGAGACATGAATGGTGGCGAAGTATGAGGC	4552
GAAAGTTGTGCCGTTAGCCAGAGTGCGTGAGACGACCTGATTGTACACCTGAAAGCCCGGGGGGAAGGATGTTGGGAAGGAGCTCCCAATGAGGCTTCCATGGGAAGGTGTACTG	4672
CTTTAGCCAGTCAAGAGGGTAGTGAACCAAGAGTCCATTGTTGGGTAAGTGGGTCGTTGATGTGAGTGTCTAAGCCTGTCTAGTGTCCGTATACAGTATCTATGGCTACACGCCCT	4792
ACTCATATGGGCGAGAAATCGAATCACCCTTACCAATGAACCCAGGAAGAAGATTC	4852

Fig. 2. Nucleotide and deduced amino acid sequences of the *AsrglA* gene.

Numbers on the right refer to the nucleotide sequence (negative numbers refer to nucleotides upstream of the *AsrglA* ATG translation start codon) and amino acid sequence. Intron sequences are denoted in lower-case letters. The TATA box and a polyadenylation signal-like sequence are double underlined. Asterisk (*) marks the translation stop codon. The N-terminal amino acid sequence analyzed chemically is underlined. The *AsrglA* DNA sequence is available in the DDBJ/EMBL/GenBank databases under accession number AB716957.

1)	MLSRTILFSTSLWARVANAAFGITTSDDSYVIDAGSANPLKFTVSRSSCDITSINYGSELQYSGTGSHIGSGGLGSADVSAVEDGDYIK	90
2)	MLKASLLSFVAFT-AQVAHAAFGITTSAYVIDTNAPNQLKFTVSRSSCDITSIHYGTELQYSSQGSHIGSGGLGSATVTATQSGDYIK	89
3)	MFQGTGLLLSLSLWTKVAAAFGITTSYVIDAGSANPLKFTVSRSSCDITSINYSGTELQYASKGSHISSGLGRATVSATQDGDYIK	90
4)	MLSRTILFSTSLWVRVANAAFGITTSDDSYVIDAGSANPLKFTVSRSSCDITSINYGSELQYSGTGSHIGSGGLGSADVSAVEDGDYIK	90
5)	MLSATLLLLFLPSWARVYAAFGITTSYVIDANSPLKFTVNRNSCDITSINFYGAELQYQGTGSHIGSGGLGSASVSATQSGDYIK	90
6)	MLFRTSLLSLLSLAAGVFNADFGITTNDDSYVINANSPLSLVFTVDRGSCDITSIVHYGTELQYSGKGSHIGSGGLGTATVSATKSGDYIK	90
7)	MLSKTFLSSAVLWARVANAAFGITDNSSYTDANSPLKFTVNKSSCDITSIVYGGSEFYSGKGSHIGSGGLGSATVSATQSGDYIK	90
8)	MLSKTSLLSLLSLAAGVFNADFGITTNDDSYVINANSPLSLVFTVDRGSCDITSIVHYGTELQYSGKGSHIGSGGLGTATVSATKSGDYIK	90
9)	MLSRTILFSTSLWVRVANAAFGITTSDDSYVIDAGSANPLKFTVSRSSCDITSINYGSELQYSGTGSHIGSGGLGSADVSAVEDGDYIK	90
10)	MLSKALFFSSLPLWAKVASAAGGITTDSSYTDAGSPNPLKFTVSRSSCDITSINYGSELQYQSKGSHIGSGGLGSASVSATQSGDYIK	90
* * * * *		
▽ ▽		
1)	VTCDDTLTQYFVVHNGDSVIHMATYITEEPSVGELRFIARLNSSELLPNEEPFGDVSTTSGGEAIEGSDVFLVDGETRSKFYSSQRFIDD	180
2)	VTCVDTLTQYFVVHNGDPI IHMATYITAEP SIGELRFIARLNSDLLPNEEPFGDVSTTADGTAIEGSDVFLVGSETRSKFYSSERFIDD	179
3)	VTCATSTLTHYFVVHNGDST IHMATHITAEP SIGELRFIARLNSDVLNNEEPFGDVSTTAGGTAIEGSDVFLVNGKTRSKFYSSQRFIDD	180
4)	VTCDDTLTQYFVVHNGDSVIHMATYITEEPSVGELRFIARLNSSELLPNEEPFGDVSTTSGGEAIEGSDVFLVDGETRSKFYSSQRFIDD	180
5)	VTCSTSTLTHYFVVHNGDPI IHMATYITAEP SIGELRFIARLNSLLPNEEPFGDVSTTSGGTAIEGSDVFLVNGQTRSKFYSSERFIDD	180
6)	VTCETDTLTQYFVVHNGDPI IHMATYITEEPSIGELRFIARLNPDELNNEEPFGDVSTTAGGEAIEGSDVFLVDGETRSKFYSSQRFIDD	180
7)	VTCDTSSLTHYFVVHNGDPI IHMATYITAEPDIGELRFIARLNSLLPNEEPFGDVSTTSGGSAIEGSDVFLVNGETRSKFYSSERFIDD	180
8)	VTCETDTLTQYFVVHNGDPI IHMATYITEEPSIGELRFIARLNSDVLNNEEPFGDVSTADGTAIEGSDVFLVDGETRSKFYSSQRFIDD	180
9)	VTCDDTLTQYFVVHNGDSVIHMATYITEEPSVGELRFIARLNSSELLPNEEPFGDVSTTSGGEAIEGSDVFLVDGETRSKFYSSQRFIDD	180
10)	VTCDDTLTQYFVVHNGDPI IHMATYITAEP SIGELRFIARLNSLLPNEEPFGDVSTTAGGSAIEGSDVFLVNGQTRSKFYSSQRFIDD	180
* * * * *		
▽		
1)	QRHCVAGDAHRVCMILNQYETSSGGPFHRDINSNNGGSSYNLYWYMNSGHVQTEDRRQGLHGPYSMYFSRSGTPSTDDITDTSFFANLDIK	270
2)	QRHCAGDAHRVCMILNQYESSGGPFHRDINSNNGGSSYNLYWYMNSGHVQTESYRMGLHGPYSMYFSRSGTPSTDDITDTSFFADLDIK	269
3)	QRHCISGSAHRVCMILNQYESSGGPFHRDIDANNNGGFFNALYWYMNSGHVQTEPYRMGLHGPYSMYFSRSGMPGTNIDTTSFFANLDIK	270
4)	QRHCVAGDAHRVCMILNQYETSSGGPFHRDINSNNGGSSYNLYWYMNSGHVQTEDRRQGLHGPYSMYFSRSGTPSTDDITDTSFFANLDIK	270
5)	HRHCVSAGSAHRVCMILNQYESSGGPFHRDINTNNGGQYNALYWYMNSGHVQTEANRMGLHGPYSMYFSRSGTPGTNIDTTSFFANLDIK	270
6)	QRHCAGDEHRVCMILNQYETSSGGPFHRDINSNNGGSSYNLYWYMNSGHVQTESYRMGLHGPYSMYFSRSGTPSTDDITDTSFFADLDIEG	270
7)	HRHCISGSAHRVCMILNQYESSGGPFHRDINSNNGGDYNALYWYMNSGHVQTESFRTGLHGPYSMYFSRSGTPSTNIDTTSFFASLGIK	270
8)	QRHCAGDEHRVCMILNQYETSSGGPFHRDINSNNGGDYNALYWYMNSGHVQLESYRMGLHGPYSMYFSRSGTPSTDDITDTSFFADLDIEG	270
9)	QRHCVAGDAHRVCMILNQYETSSGGPFHRDINSNNGGSSYNLYWYMNSGHVQTESYRMGLHGPYSMYFSRSGTPSTDDITDTSFFANLDIK	270
10)	QRHCISGSAHRVCMILNQYESSGGPFHRDINSNNGGDYNALYWYMNSGHVQTESYRMGLHGPYSMYFSRSGTPGTNIDTTSFFANLDIK	270
* * * * *		
1)	YVAADGRGTVSGTASGADSSFKWVWHYNDAAQYWTYTSSDGSFTSPAMKPGDYMVYQGEYKVAETSVSVTAGSSTSKDISGSVETGD	360
2)	YVAASGRGKVAGTASGADSSMDWVWHYNDAAQYWTYTSSDGSFTSPAMKPGTYTMVYQGEYAVATSSVTSAGSTTKNISGSVKTGT	359
3)	YVPASGRGTVTGKASGADSRMKWVWHYNDAAQYWTYTASDGSFTSPAMKPGTYTMVYQGEYKVAETSVTVRAGSKITKNISGSVKTGT	360
4)	YVAADGRGTVSGTASGADSSFKWVWHYNDAAQYWTYTSSDGSFTSPAMKPGDYMVYQGEYKVAETSVSVTAGSSTSKDISGFVETGD	360
5)	YVPDARGKVSAGKASGADSTFKWVWHYNDAAQYWTYTASDGSFTSPAMKPGTYTMVYQGEYKVAETSVSVTAGSSTTKNISGSVTTGK	360
6)	YVAEADRGTVSGTASGADSSFDWVWHYNDAAQYWTYTSSDGSFTSPAMKPGTYTMVYQGEYVAVATSEVTVSAGSSTTKNISGSVKTGT	360
7)	YVAANGRGTVTGKASGADSSMDWVWHYNDAAQYWTYTASDGSFTSPAMKPGTYTMKYQGEFPVAETTVTSAGSSTTKNISGSVKTGT	360
8)	YVAESGRGTVSGTASGADSSFDWVWHYNDAAQYWTYTSSDGSFTSPAMKPGTYTMVYQGEYVAVATSEVTVSAGSSTSKDISGSVETGT	360
9)	YVATDGRGTVSGTASGADSSFKWVWHYNDAAQYWTYTSSDGSFTSPAMKPGDYMVYQGEYKVAETSVSVTVGSSTSKDISGSVETGD	360
10)	YVPASGRGTVTGKASGADSNFKWVWHYNDAAQYWTYTASDGSFTSPAMKPGTYTMAYYQGEYRVAETSVTVSAGSSTTKNISGSVKTGT	360
* * * * *		
1)	TIFKIGDWDGTPGFRNAENQLRMHPSDSRMSSWGPLYTYVGSSSLDFFPMAAFKGVNDPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	450
2)	TIFKIGEDWGQPTGFRNAENQLRMHPSDSRMSSWGPLYTYVGSSSLDFFPMAVFKSVNNPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	449
3)	TIFKIGEDWGQPTGFRNADKQLRMHPSDSRMAAWGPLYTYVGRSAPSDFFPMAVFKSVNNPVTIKFTASAAQTAAATLRIGTTLSFAGGRP	450
4)	TIFKIGDWDGTPGFRNAENQLRMHPSDSRMSSWGPLYTYVGSSSLDFFPMAAFKGVNDPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	450
5)	TIFKIGEDWGQPTGFRNAENQLRMHPSDSRMSSWGPLYTYVGSSSLDFFPMAIFKSVNSPVTIKFTASSQTGAATLRIGTTLSFAGGRP	450
6)	TIFKIGDWDGQPTGFRNAENQLRMHPSDSRMSDWGPLYTYVGSSSLDFFPMAVFKAVNDPVTIKFTATSDQTGAATLRIGTTLSFAGGRP	450
7)	TIFKIGEDWGQPTGFRNADKQLRMHPSDSRMSDWSS-TYTVGSSSLDFFPMAVFKSVNNPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	449
8)	TIFKIGDWDGQPTGFRNAENQLRMHPSDSRMSDWGPLYTYVGSSSLDFFPMAIFKSVNSPVTIKFTATSDQTGAATLRIGTTLSFAGGRP	450
9)	TIFKIGDWDGTPGFRNAENQLRMHPSDSRMSSWGPLYTYVGSSSLDFFPMAAFKGVNDPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	450
10)	TIFKIGDWDGQPTGFLNADKQLRMHPSDSRMSSWGPLYTYVGSSSVGSFPMALFKSVNSPVTIKFTATSAQTGAATLRIGTTLSFAGGRP	450
* * * * *		
▽ ▽		
1)	QATINDYEGSAPSAPTNLDSRGVTRGAYRGLGEVYDVSIPSGTIVEGENTITISVISGSSGDEFLAPNFIFDCVELFQ---	528 <i>A. sojae</i>
2)	QATINSYTGSAAPTNLDSRGVTRGAYRGLGEVYDVSIPSGTIVAGTNTITINVISGSSGDTYLSPNFIFDCVELFQ---	527 <i>A. aculeatus</i>
3)	QAKINSYTAAPAPKNDLSRGVTRGAYRGLGEVYDVAIPAGTIVAGTNTITISVVSNGSGEKLSPNFVFDVELFQ---	528 <i>A. clavatus</i>
4)	QATINDYEGSAPSAPTNLDSRGVTRGAYRGLGEVYDVNIPSGTIVEGENTITISVISGSSGDEFLAPNFIFDCVELFQ---	528 <i>A. flavus</i>
5)	QVTVNSWTGPIPSAPKDLNSRGVTRGAYRGLGEVYDVAIPAGTIVAGTNTITISVVSNGSGDAFLSPNFIFDCVELFQ---	528 <i>A. fumigatus</i>
6)	QATINDYEGSAPSAPTNLDSRGVTRGAYRGLGEVYDVNIPSGTIVEGENTITISVISGSSGDEFLSPNFIFDCVELFQ---	528 <i>A. kawachii</i>
7)	QATINSYTGPAAPSAPTNLDSRGVTRGAYRGLGEVYDVSVPAITVAGTNTITISVISGSSGDAFLSPNVFDCIELFQ---	527 <i>A. nidulans</i>
8)	QATINDYEGSAPSAPTNLDSRGVTRGAYRGLGEVYDVSVPEGTIVEGENTITISVISGSSGDDFLSPNFDAVFIILVDN	531 <i>A. niger</i>
9)	QATINDYEGSAPSAPTNLDSRGVTRGAYRGLGEVYDVNIPSGTIVEGENTITISVISGSSGDEFLAPNFIFDCVELFQ---	528 <i>A. oryzae</i>
10)	QATINSYTGPTAPSAPTNLDSRGVTRGAYRGLGEVYDVSIPAGTIVAGTNTITINVISGSSGDDFLSPNFVSAYPFLTLMRC	531 <i>A. terreus</i>
* * * * *		

Fig. 3. Alignment of the AsRglA amino acid sequence with those of rhamnogalacturonan lyases from other *Aspergilli*.

Asterisks (*) indicate amino acids conserved among all ten sequences. The catalytic and substrate-binding residues are indicated by solid and open arrowheads, respectively.

aculeatus and *A. niger*.

For amplification of the 5'- and 3'- regions of the RHG1, four specific primers, termed rhgB5, rhgB6, rhgB7 and rhgB8, were synthesized based on the nucleotide sequence of RHG1. Through cassette-ligation mediated PCR (CLM-PCR) with a *Sa*I or an *Eco*RI cassette-ligated genomic DNA as the template, a 718-bp fragment for the 5'-region designated RHG2 or a 3,461-bp fragment for the 3'-region designated as RHG3 was amplified, as described in Materials and Methods. RHG2 had the nucleotide sequence of the 5'-region of RHG1 and the ATG start codon of the *AsrglA* gene. RHG3 had a nucleotide sequence of the 3'-region of RHG1 and the TAA stop codon of the *AsrglA* gene. Based on the sequences of the RHG 1, RHG 2 and RHG 3, the entire nucleotide sequence of the *AsrglA* gene was determined (Fig. 2). To define the intron/exon structure of the *AsrglA* gene, five RT-PCR-derived cDNAs were cloned and sequenced.

The coding region consisted of 2,245 bp and was interrupted by 11 introns ranging in size from 48 bp to 74 bp (Fig. 2). The sequence encoded a polypeptide of 528 amino acid residues with a molecular mass of 56,869 Da. The N-terminal amino acid sequence of the purified recombinant AsRglA protein from the *A. oryzae* strain transformed with the *AsrglA* gene, as described below, was in complete agreement with that deduced from Ala-21 to Ser-30. Analysis using the SignalP 4.0 program (i) predicted that the first 20 N-terminal amino acids of AsRglA function as a signal sequence. The molecular mass of the mature AsRglA was calculated to be 54,603 Da.

The 5'- and 3'-noncoding regions of the *AsrglA* gene were searched for various consensus sequences. A potential TATA box at -76 to -72 and a polyadenylation signal-like sequence, AATAAG, at +2,386 to +2,391, referring to the translation start site as +1 were found within the region we determined.

Comparison of amino sequence of *AsrglA* with those of other *Aspergilli* rhamnogalacturonases The deduced amino acid sequence of *AsrglA* was compared with those of rhamnogalacturonan lyases from other *Aspergilli*. Overall, AsRglA showed significant sequence identity to rhamnogalacturonan lyases from other *Aspergilli*. Alignment of rhamnogalacturonan lyase sequences from *A. sojae*, *A. aculeatus*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus kawachii*, *Aspergillus nidulans*, *A. niger*, *A. oryzae* and *Aspergillus terreus* is shown in Fig. 3. AsRglA showed 83.1% identity to *A. aculeatus* RhgB, 78.8% to *A. clavatus* rhamnogalacturonase B (ACLA_054660) (Fedorova *et al.*, 2008), 98.7% to *A. flavus* rhamnogalacturonase B (AFLA_041820A) (Payne *et al.*, 2006), 81.1% to *A. fumigatus* rhamnogalacturonase B (AFUA_4G03780) (Nierman *et al.*,

2005), 85.4% to *A. kawachii* rhamnogalacturonate lyase A (Futagami *et al.*, 2011), 85.4% to *A. nidulans* RGaseB (AN7135.2) (Galagan *et al.*, 2005), 85.2% to *A. niger* RglA (An14g001130), 98.7% to *A. oryzae* rhamnogalacturonate lyase A (AO090011000349) (Machida *et al.*, 2005), and 61.3% to *A. terreus* RglA (ATEG_02193) (ii).

Based on the results of X-ray crystallography combined with site-directed mutagenesis of *A. aculeatus* RhgB, the catalytic and substrate-binding residues were determined from the three-dimensional structure (McDonough *et al.*, 2004, Jensen *et al.*, 2010); Lys-169 and His-229 are involved in catalysis, and Arg-126, Arg-130, Arg-470 and Arg-474 are involved in substrate binding. The corresponding residues in AsRglA, are also likely involved in catalysis and substrate binding, i.e., Lys-170 and His-230 for catalysis, and Arg-127, Arg-131, Arg-471 and Arg-475 for substrate binding.

Overexpression, purification and characterization of *AsrglA* In order to characterize the enzymatic properties of the *AsrglA* gene product, the *AsrglA* gene was efficiently expressed under the control of the *A. oryzae* *taaG2* gene promoter, which is one of the strongest promoters in *A. oryzae*. An expression vector, pTARB100, comprising the *AsrglA* gene under the control of the *A. oryzae* *taaG2* gene promoter, was constructed as described in the Materials and Methods,

Fig. 4. SDS-polyacrylamide gel electrophoresis of the AsRglA secreted by *A. oryzae* transformant SS28 (A) and the purified AsRglA (B).

(A) The *A. oryzae* transformant SS28 was cultivated as described in the Materials and Methods. Culture filtrates (9 μ L) were subjected to SDS-PAGE on a 12.5% gel and stained with Coomassie Brilliant Blue. Lane 1, molecular-mass markers [rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), rabbit muscle aldolase (42.4 kDa), bovine carbonic anhydrase (30.0 kDa), and soybean trypsin inhibitor (20.1 kDa)]; lane 2, culture filtrate of *A. oryzae* transformant carrying pYRMTA100; lane 3, culture filtrate of *A. oryzae* transformant SS28.

(B) AsRglA was purified as described in the Materials and Methods and subjected to SDS-PAGE on a 12.5% gel and stained with Coomassie Brilliant Blue. Molecular-mass markers (lane 1) are as described in the legend to (A). Lane 2, purified AsRglA.

Fig. 5. Effects of pH (A) and temperature (B) on the activity (●) and stability (■) of purified AsRglA.

Reactions were carried out as described in the Materials and Methods. The values are means of three replicates with standard deviations (error bars).

and introduced into *A. oryzae* KBN616-P3. Of 30 transformants screened on the AGM agar plate, 26 transformants secreted a larger amount of rhamnogalacturonan lyase than the control strain into which the plasmid pYRMTA100 was introduced. Five high AsRglA-producing strains selected by plate assay secreted 17.3 to 69.3 U/mL (124 to 497 mg/L) of rhamnogalacturonan lyase after growth in RB medium for 4 days whereas the control strain showed no rhamnogalacturonan lyase activity. After electrophoresis of the culture supernatant on a sodium dodecyl sulfate/polyacrylamide gel, AsRglA was detected as a prominent band in the culture supernatant of a high-AsRglA-producing strain, SS28, when stained with Coomassie Brilliant Blue (Fig. 4 (A), lane 3). Furthermore, this enzyme production level (497 mg/L) was almost five times higher than that (107 mg/L) of the *A. sojae* polygalacturonase gene, *AspecA* gene, expressed under the control of the *A. oryzae* *taaG2* gene promoter (Yoshino-Yasuda *et al.*, 2011). AsRglA was purified to homogeneity from the culture supernatant of the SS28 strain by anion-exchange chromatography, as described in the Materials and Methods. AsRglA was purified 1.2-fold, with recovery of 65.4% of the initial activity and a specific activity of 139.4 U/mg of protein. The purified AsRglA had a molecular mass of 55.0 kDa (Fig. 4 (B), lane 2), which was very similar to the calculated value of 54,603 Da, as described above.

The enzymatic properties of AsRglA, including pH and temperature optima and pH and thermal stabilities, were investigated with the purified protein (Fig. 5). AsRglA had a pH optimum of 5.0 and a temperature optimum of 50°C. The pH optimum for AsRglA was lower than that reported for *A.*

aculeatus RhgB, while the temperature optimum for AsRglA was similar to that for *A. aculeatus* RhgB. AsRglA was stable in a wide range of pH from 3.0 to 7.0 and almost 100% of the activity was retained. AsRglA was stable up to 45°C and about 90% of its activity was retained. However, AsRglA was inactivated sharply at temperatures above 45°C and no enzyme activity remained at temperatures above 50°C. To our knowledge, this is the second report of a detailed experimental analysis on the fungal rhamnogalacturonan lyase, as the majority of fungal rhamnogalacturonan lyases have only been discussed based on the DNA sequence of the genes encoding their enzymes.

We are now investigating the use of the high AsRglA-producing transformant in soy sauce brewing and evaluating the effects of rhamnogalacturonan lyase on the degradation of soybean pectin.

Acknowledgments We would like to thank S. Suzuki for technical assistance.

References

- Fedorova, N.D., Khaldi, N., Joardar, V.S., Maiti, R., Amedeo, P., Anderson, M.J., Crabtree, J., Silva, J.C., Badger, J.H., Albarraq, A., Angiuoli, S., Bussey, H., Bowyer, P., Cotty, P.J., Dyer, P.S., Egan, A., Galens, K., Fraser-Liggett, C.M., Haas, B.J., Inman, J.M., Kent, R., Lemieux, S., Malavazi, I., Orvis, J., Roemer, T., Ronning, C.M., Sundaram, J.P., Sutton, G., Turner, G., Venter, J.C., White, O.R., Whitty, B.R., Youngman, P., Wolfe, K.H., Goldman, G.H., Wortman, J.R., Jiang, B., Denning, D.W. and Nierman, W.C. (2008). Genomic islands in the pathogenic

- filamentous fungus *Aspergillus fumigatus*. *PLoS Genet.*, **4**, E1000046.
- Futagami, T., Mori, K., Yamashita, A., Wada, S., Kajiwar, Y., Takashita, H., Omori, T., Takegawa, K., Tashiro, K., Kuhara, S. and Goto, M. (2011). Genome sequence of the white koji mold *Aspergillus kawachii* IFO4308, used for brewing the Japanese distilled spirit shochu. *Eukaryotic Cell*, **10**, 1586-1587.
- Galagan, J.E., Calvo, S.E., Cuomo, C., Ma, L.J., Wortman, J.R., Batzoglou, S., Lee, S.I., Bastürkmen, M., Spevak, C.C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scacciocchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G.H., Draht, O., Busch, S., D'Enfert, C., Bouchier, C., Goldman, G.H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J.H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E.U., Archer, D.B., Peñalva, M.A., Oakley, B.R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W.C., Denning, D.W., Caddick, M., Hynes, M., Paoletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M.S., Osmani, S.A. and Birren, B.W. (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature*, **438**, 1105-1115.
- Heldt-Hansen, H.P., Kofud, L.V., Budolfson, G., Nielson, P.M., Huttel, S., and Bladt, T. (1996). Application of tailor made pectinase, p.463- 474. In J. Visser and A.G.J. Voragen (ed.), *Pectins and Pectinases*. Elsevier Science B. V., Amsterdam, The Netherlands.
- Isegawa, Y., Sheng, J., Sokawa, Y., Yamanishi, K., Nakagomi, O. and Ueda, S. (1992). Selective amplification of cDNA sequence from total RNA by cassette-ligation mediated polymerase chain reaction (PCR): application to sequencing 6.5 kb genome segment of hantavirus strain B-1. *Mol. Cell Probes*, **6**, 467-475.
- Jensen, M.H., Otten, H., Christensen, U., Borchert, T.V., Christensen, L.L.H., Lasen, S. and Leggio, L.L. (2010). Structural and biochemical studies elucidate the mechanism of rhamnogalacturonan lyase from *Aspergillus aculeatus*. *J. Mol. Biol.*, **404**, 100-111.
- Kikuchi, T. (1977). Behavior and role of soybean polysaccharides on soy sauce fermentation. *Nippon Shoyu Kenkyujo Zasshi*, **3**, 154-159 (in Japanese).
- Kitamoto, N., Kimura, T., Kito, Y., Ohmiya, K. and Tsukagoshi, N. (1995). The nitrate reductase gene from a *shoyu koji* mold, *Aspergillus oryzae* KBN616. *Biosci. Biotechnol. Biochem.*, **59**, 1795-1797.
- Kofod, L.V., Kauppinen, S., Christgau, S., Anderson, L.N., Heldt-Hansen, H.P., Dörreich, K. and Dalbøge, H. (1994). Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. *J. Boil. Chem.*, **269**, 29182-29189.
- Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., Abe, K., Gomi, K., Horiuchi, H., Kitamoto, K., Kobayashi, T., Takeuchi, M., Denning, D. W., Galagan, J.E., Nierman, W.C., Yu, J., Archer, D.B., Bennett, J.W., Bhatnagar, D., Cleveland, T.E., Fedorova, N. D., Gotoh, O., Horikawa, H., Hosoyama, A., Ichinomiya, M., Igarashi, R., Iwashita, K., Juvvadi, P.R., Kato, M., Kato, Y., Kin, T., Kokubun, A., Maeda, H., Maeyama, N., Maruyama, J., Nagasaki, H., Nakajima, T., Oda, K., Okada, K., Paulsen, I., Sakamoto, K., Sawano, T., Takahashi, M., Takase, K., Terabayashi, Y., Wortman, J.R., Yamada, O., Yamagata, Y., Anazawa, H., Hata, Y., Koide, Y., Komori, T., Koyama, Y., Minetoki, T., Suharnan, S., Tanaka, A., Isono, K., Kuhara, S., Ogasawara, N. and Kikuchi, H. (2005). Genome sequencing and analysis of *Aspergillus oryzae*. *Nature*, **438**, 1157- 1161.
- McDonough, M.A., Kadirvelraj, R., Harris, P., Pousen, J.-C., N. and Larsen, S. (2004). Rhamnogalacturonan lyase reveals a unique three-domain modular structure for polysaccharide lyase family 4. *FEBS Lett.*, **565**, 188-194.
- Mutter, M., Beldman, G., Schols, H.A. and Vorgaen, A.G.J. (1994). Rhamnogalacturonan α -L-rhamnopyranohydrolase. A novel enzyme specific for rhamnogalacturonan regions of pectin. *Plant Physiol.*, **106**, 241-250.
- Mutter, M., Colquhoun, I.J., Schols, H.A., Beldman, G. and Voragen, A.G.J. (1996a). Rhamnogalacturonan B from *Aspergillus aculeatus* is a rhamnogalacturonan α -L-rhamnopyranosyl-(1,4)- α -D-galactopyranosyluronide lyase. *Plant Physiol.*, **110**, 73-77.
- Mutter, M., Renard, C.M.G.C., Beldman, G., Schols, H.A. and Voragen, A.G.J. (1996b). Characterization of RG degradation products of new RGases using RG-rhamnohydrolase and RG-galacturonohydrolase. In "Pectins and Pectinases," ed. by J. Visser and A.G.J. Voragen. Elsevier Science B.V., Amsterdam, pp. 263-274.
- Mutter, M., Colquhoun, I.J., Beldman, G., Schols, H.A., Bakx, E.J. and Voragen, A.G.J. (1998). Characterization of recombinant rhamnogalacturonan α -L-rhamnopyranosyl-(1,4)- α -D-galactopyranosyluronide lyase from *Aspergillus aculeatus*. *Plant Physiol.*, **117**, 141-152.
- Nelson, M.A., Merino, S.T. and Metzenberg, R.L. (1997). A putative rhamnogalacturonase required for sexual development of *Neurospora crassa*. *Genetics*, **146**, 531-540.
- Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., Arroyo, J., Berriman, M., Abe, K., Archer, D.B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulsen, R., Davies, R., Dyer, P.S., Farman, M., Fedorova, N., Fedorova, N., Feldblyum, T.V., Fischer, R., Fosker, N., Fraser, A., García, J. L., García, M.J., Goble, A., Goldman, G.H., Gomi, K., Griffith-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuchi, H., Huang, J., Humphray, S., Jiménez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Latgé, J.P., Li, W., Lord, A., Lu, C., Majoros, W.H., May, G.S., Miller, B.L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O'Neil, S., Paulsen, I., Peñalva, M.A., Perte, M., Price, C., Pritchard, B.L.,

- Quail, M.A., Rabbino-witsch, E., Rawlins, N., Rajandream, M.A., Reichard, U., Renauld, H., Robson, G.D., Rodriguez de Córdoba, S., Rodríguez-Peña, J.M., Ronning, C.M., Rutter, S., Salzberg, S.L., Sanchez, M., Sánchez-Ferrero, J.C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaiia, F., Turner, G., Vazquez de Aldana, C.R., Weidman, J., White, O., Woodward, J., Yu, J.H., Fraser, C., Galagan, J.E., Asai, K., Machida, M., Hall, N., Barrell, B. and Denning, D.W. (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature*, **438**, 1151-1156.
- Payne, G.A., Nierman, W.C., Wortman, J.R., Pritchard, B.L., Brown, D., Dean, R.A., Bhatnagar, D., Cleveland, T.E., Machida, M. and Yu, J. (2006). Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. *Med. Mycol.*, **44**, 9-11.
- Pel, H.J., de Winde, J.H., Archer, D.B., Dyer, P.S., Hofmann, G., Schaap, P.J., Turner, G., de Vries, R.P., Albang, R., Albermann, K., Andersen, M.R., Bendtsen, J.D., Benen, J.A., van den Berg, M., Breestraat, S., Caddick, M.X., Contreras, R., Cornell, M., Coutinho, P.M., Danchin, E.G., Debets, A.J., Dekker, P., van Dijck, P.W., van Dijk, A., Dijkhuizen, L., Driessen, A.J., d'Enfert, C., Geyssens, S., Goosen, C., Groot, G.S., de Groot, P. W., Guillemette, T., Henrissat, B., Herweijer, M., van den Hombergh, J.P., van den Hondel, C.A., van der Heijden, R.T., van der Kaaij, R.M., Klis, F.M., Kools, H.J., Kubicek, C.P., van Kuyk, P.A., Lauber, J., Lu, X., van der Maarel, M.J., Meulenberg, R., Menke, H., Mortimer, M.A., Nielsen, J., Oliver, S.G., Olsthoorn, M., Pal, K., van Peij, N.N., Ram, A.F., Rinas, U., Roubos, J.A., Sagt, C.M., Schmoll, M., Sun, J., Ussery, D., Varga, J., Vervecken, W., van de Vondervoort, P.J., Wedler, H., Wosten, H.A., Zeng, A.P., van Ooyen, A.J., Visser, J. and Stam, H. (2007). Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat. Biotechnol.*, **25**, 221-231.
- Petesen, T.N., Kauppinen, S. and Larsen, S. (1997). The crystal structure of rhamnogalacturonase A from *Aspergillus aculeatus*: a right-handed parallel β -helix. *Structure*, **5**, 533-544.
- Tsukagoshi, N., Furukawa, M., Nagaba, H., Kirita, N., Tsuboi, A., and Udaka, S. (1989). Isolation of a cDNA encoding *Aspergillus oryzae* taka-amylase A: evidence for multiple related genes. *Gene*, **84**, 319-327.
- Willats, W.G., McCartney, L., Mackie, W. and Knox, J.P. (2001). Pectin; cell biology and prospects for functional analysis. *Plant Mol. Biol.*, **47**, 9-27
- Yoshino-Yasuda, S., Kato, M. and Kitamoto, N. (2011). Sequence analysis and heterologous expression of polygalacturonase gene (*AspecA*) from a *shoyu koji* mold, *Aspergillus sojae* KBN1340. *Food Sci. Technol. Res.*, **17**, 579-584.

URL Cited

- i) <http://www.cbs.dtu.dk/services/SignalP/> (2012.5.5)
- ii) http://www.broadinstitute.org/annotation/genome/aspergillus_group/ (2012.5.5)