Journal of Bacteriology and Virology 2014. Vol. 44, No. 2 p.152 – 161
http://dx.doi.org/10.4167/jbv.2014.44.2.152

Original Article

Aspergillus oryzae S-03 Produces Gingipain Inhibitors as a Virulence Factor for *Porphyromonas gingivalis*

Narandalai DANSHIITSOODOL, Hideyuki YAMASHITA, Masafumi NODA, Takanori KUMAGAI, Yasuyuki MATOBA and Masanori SUGIYAMA*

Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical and Health Sciences, Hiroshima University; Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

Oral infection with *Porphyromonas (P.) gingivalis* causes periodontitis that is manifested by the destruction of gingival connective tissues. Although a few types of antibiotics are effective against the infection, its use induces the appearance of drug-resistant bacteria. The present study shows that the fermented product of *Aspergillus (A.) oryzae* S-03, cultivated on the fat-removed soybean, inhibits the cell growth of the *P. gingivalis*. Likewise, the fermented product of the S-03 strain cultured for 26~42 h displays an inhibitory activity to gingipain as a virulence factor of *P. gingivalis*. The activity is not lost even with heat treatment at 100℃ for 15 min. We also demonstrate that the S-03 strain exhibits high protease activity. In addition, the strain does not produce aflatoxin because of the loss of a regulatory gene, *aflR*, necessary for the toxin biosynthesis.

Key Words: *Apergillus oryzae*, Gingipain, Inhibitor, Koji

INTRODUCTION

In mycology, a genus of filamentous fungi, *Aspergillus*, is extremely diverse and widely distributed. There are over 185 known species of the genus *Aspergillus*, of which there are species which produce aflatoxins, such as *A. flavus* and *A. parasiticus*. Aflatoxins are classified as a group of highly oxygenated polyketide-derived furanocoumarins causing adverse effects such as acute hepatic toxicity in human (1~3). Despite this fact, sufficient varieties of *Aspergillus* species are useful for the food and biotechnology industries. Bhanja *et al*. (4) has demonstrated that the fermented products of wheat grain with *A. oryzae* and *A. awamori* enhance the antioxidant activity of wheat. It has also been shown that oleic compounds produced by five different strains of *A. oryzae* inhibit cholesterol synthesis in the human hepatic T9A4 cells *in vitro* (5). *A. oryzae* is essentially used to ferment soybeans and wheat or rice to make a soybean paste, called "miso" in Japanese, and soy sauce, as well as to saccharify rice for the production of sake and other distilled spirits. A recent report (6) has indicated that during the fermentation process for miso production, hydrolyzation of casein by *A. oryzae* gives rise to anti-hypertensive peptides that inhibit the angiotensin-I converting enzyme.

Periodontal disease affects up to 40% of the adult popu-

Received: March 10, 2014/ Revised: March 14, 2014/ Accepted: March 17, 2014
*Corresponding author: Masanori Sugiyama, Ph.D., Professor. Department of Molecular Microbiology and Biotechnology, Graduate School of Biomedical and Health Sciences, Hiroshima University. Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan.
Phone: +81-82-257-5280, Fax: +81-82-257-5283, e-mail: sugi@hiroshima-u.ac.jp
**We thank Mr. Matsunosuke Higuchi, the president of Higuchi Matsunosuke Shoten Co.Ltd, Japan, for his donation of *A. oryzae* strains.
We thank Dr. Osamu Yamada, National Research Institute of Brewing, Japan for supporting analysis of aflatoxin-encoding gene cluster.

lation in developed or developing countries. *Porphyromonas (P.) gingivalis*, which is a gram-negative black-pigmented anaerobic bacterium, is detected in the microflora of subgingival plaques. In recent years, there have been many reports dealing with the physiology and the functional properties of this bacterium in the pathogenesis of adult periodontitis (7, 8). A multiple virulence factor of *P. gingivali*s involved in tissue destruction, evasion of host defenses, and invasion includes fimbriae, hemagglutinins, and lypo-polysaccharides (LPS). Another aspect of the process of degrading connective tissues as well as host defense macromolecules that has been well characterized is trypsin-like cysteine proteases, referred as gingipains. Two types of gingipains have been found: one is arginine specific (Arg-gingipains or Rgp), and another is lysine specific (Lys-gingipains or Kgp). It has been suggested that gingipains are responsible for at least 85% of the proteolytic activity of this bacterium that develops edema, neutrophil infiltration, and bleeding due to the degradation of fibrinogen in human plasma. Gingipains may be assembled as Kgp-RgpA complexes that are closely associated with cell surfaces or they may be liberated into the growth medium. Both gingipains also noncovalently complexed with 44 kDa hemagglutinins (9~13).

Currently, the use of antibiotics is widely accepted for treating *P. gingivalis* infection (14). Unfortunately, this treatment increasingly encounters the resistant strains and causes side effects of antibiotics such as epigastric pain and severe diarrhea. With respect to the disease, Kadowaki *et al*. (15) have demonstrated high suppression profiles of synthetic peptide KYT-1 and KYT-36 with a concentration of less than 1 μM to gingipain activities, although issues are still pending regarding the safety of these peptides for human use. Similarly, leupeptin and antipain, two low-molecular-weight peptides, acts on a broad spectrum of cellular serine and cysteine proteases and displays high potential for inhibiting the activity of gingipains (16, 17). At the same time, it has been reported that leupeptin induces certain morphological changes on cerebellum granule neurons *in vitro* (18). Much effort has thus been made in the search for more-efficient and less-invasive treatments against the pathogenicity of this bacterium. These include peptides from a chymosin digest of casein, green tea catechins, and polyphenolic compounds from cranberries (19~22). Protegrins isolated from porcine leukocytes have been shown also to inhibit the growth of *P. gingivalis in vitro* (23).

Although many studies describe the biological and industrial uses of koji, studies regarding its medical uses, such as developing substances effective against pathogenic bacteria, are few (24). In the present study, we show that when cultivated onto fat-removed soybeans, *A. oryzae* S-03 produces inhibitors to the cell growth of *P. gingivalis* and to gingipain as a virulence factor of *P. gingivalis*.

MATERIALS AND METHODS

The preparation of koji

Slightly polished white rice was soaked overnight in distilled water at room temperature. After the soaking, the grains were drained and then steamed for 30 min. The preparation was transferred to a culture flask, autoclaved for 20 min at 121℃, and, once cooled to 35℃, the resulting grains were inoculated with the conidia of *A. oryza*e S-03, which was provided by Higuchi Matsunosuke Shoten Co., Ltd. (Osaka, Japan), at 30℃ for 20 h in a humid growth chamber. The grains inoculated with the conidia of the S-03 strain were then transferred to a Petri dish and cultured for approximately 100 h. Conidia of the culture were collected through double filtration with 100- and 200-mesh nylon membranes.

Fat-removed soybean (Nisshin OilliO Group, Japan; water 13%, protein 49%, ash 6.5%, and fat 0.9%) were soaked in its 0.85 volume of distilled water for 1 h and autoclaved at 121℃ for 20 min. After cooling to 35℃, they were inoculated with *A. oryzae* S-03 conidia, at 5×10^5 colony-forming units (CFU)/g. Cultivation was carried out at 30℃ for 24 h, then at 25℃ for 18 h.

Genetic analysis of *A. oryzae* S-03 for aflatoxin production

A. oryzae S-03 was cultured on DP medium (1% peptone, 2% dextrose, 0.5% KH_2PO_4, and 0.5% $MgSO_4$)

and incubated at 30℃ for 72 h. Chromosomal DNA was extracted as described previously (25). ITS4 and ITS5 primers were used to amplify (GeneAmp PCR System 9700, ABI, US). The QIAquick PCR Purification Kit (Qiagen, US) was used to clean up amplified fragments, and this was next applied to direct sequencing (ABI 3100 Avant Genetic Analyzer) using the ITS1 and ITS4 primers. All primers used in this study to amplify several structural genes are listed in Table 1.

The condition consisting of 25 cycles of 95℃ for 20 sec, 60℃ for 30 sec, and 68℃ for 45 sec was used for the PCR reaction of the ITS primers. However, PCR conditions for *aflR* were 95℃ for 20 sec, 60℃ for 30 sec, and 68℃ for 90 sec. The resulting amplified products were confirmed by 0.8% (w/v) agarose gel electrophoresis.

For Southern analysis, the *A. oryzae* S-03 genomic DNA, which was completely digested by *Pst*I, was transferred to a HyBond N+ membrane (GE Healthcare, US). *AflR* was used as a probe. Southern blotting was performed using a PCR DIG Probe Synthesis Kit (Roche Diagnostic, Germany) according to the manufacturer's instructions.

Assays of α-amylase and protease produced by *A. oryzae* S-03

For enzyme analysis, 20 g of the fat-removed soybean koji fermented with *A. oryzae* S-03 was extracted with 100 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 0.5% (w/v) of NaCl for 3 h at room temperature. The resulting supernatant was filtered through a filter paper (Whatman No. 2) and dialyzed overnight against the same buffer at 4℃. The dialysate was used as a crude enzyme solution to measure the activities of amylase or protease. The activity of α-amylase was measured with iodine. One unit of the enzyme activity (the Wohlgemuth unit) was defined as the amount of α-amylase hydrolyzing 1% starch into noncolored reaction products per minute at 40℃ and pH 5.0. Protease activity was measured using 2% (w/v) milk casein as a substrate, according to a book "Official method of analysis of National Tax Agency, 4th ed., The Brewing Society of Japan, Tokyo (1993) (in Japanese)". Briefly, the enzyme solution was added to a 2% (w/v) milk casein (Calbiochem) solution in McIlvaine buffer (pH 6.0) and incubated for 30 min at 40℃. The enzyme reaction was stopped by the addition of 0.4 M trichloroacetic acid and then filtrated with No. 131 filter paper (Advantec, Japan). The filtrate was well mixed with 0.4 M Na_2CO_3 and Folin's reagent, and the absorbance of the reaction mixture was measured at 660 nm. The protease activity was expressed as mg of tyrosine contained in peptides liberated from 1g of koji per hour.

Preparation of the gingipain inhibitor

A solid mass of the fat-removed soybean koji was combined with 3 volumes of distilled water and gently agitated at room temperature for 1 h. Subsequently, after the suspension was centrifuged at 13,500 x g for 15 min, the resulting supernatant fluid was filtered with a 0.2-μm membrane filter (DISMIC-13cp, Advantec, Japan). Furthermore, the ultrafiltration membrane cutting off the molecular weight of 3,000 Da (MWCO 3K, Millipore, US) was used to fractionate the solute with a high yield. The permeate fraction was used to further purify the gingipain inhibitors. The obtained permeate was extracted twice by adding an equal volume of acetone, and, after centrifugation for 10 min at 6,000 x g, the solid material was subsequently discarded, and the liquid extract was freeze-dried and kept at -20℃ until use.

Cultivation of *P. gingivalis* and assay of antibacterial activities of the bacterium

P. gingivalis W83 was grown on blood agar supplemented with hemin and vitamin K (Kyokutou Seiyaku, Japan) under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37℃. The culture was added to Tryptic Soy Broth (TSB) supplemented with 0.5% yeast extract, 5 μg/ml hemin, 1 μg/ml menadione, and 0.01% dithiothreitol incubated anaerobically at 37℃. The cultures were incubated for 24 h or for times appropriate for the experimental design. The antibacterial activity produced by cultivation of *A. oryzae* S-03 on the fat-removed soybean was determined by an agar diffusion method.

Purification of gingipain from *P. gingivalis*

The culture of *P. gingivalis* W83, which was grown for 48 h, was centrifuged at 14,000 x g for 20 min at 4℃, and the cell-free culture supernatant was obtained. The supernatant fluid was added to the same volume of acetone, and the resulting precipitate was suspended in a buffer containing 0.05 M Bis-Tris, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.02% NaN_3 at pH 6.8. Prior to its application onto gel filtration chromatography, the solution was dialyzed overnight against the same buffer. The clear supernatant was applied to a HiLoad 26/600 Superdex 200 column equilibrated with the same buffer and fractionated at a flow rate of 20 ml/h. Active fractions with low-molecular-weight enzymes were pooled and dialyzed for 24 h against a 50 mM Bis-Tris buffer (pH 6.8) containing 1 mM $CaCl_2$. DE-52 (Whatman Co., UK) anion exchange chromatography was performed for an initial step of the low molecular weight gingipain RgpB according to the methods described previously (12, 26). On the other hand, fractions containing high-molecular-weight RgpA-Kgp protease complexes were pooled and dialyzed against a 50 mM Tris-HCl buffer (pH 8.0). The resulting protein solution was applied on a Q-Sepharose (GE Healthcare, US) anion exchange column equilibrated with a 50 mM Tris-HCl buffer (pH 8.0). The enzyme was eluted with a linear gradient of NaCl (from 0 to 0.5 M) in a 50 mM Tris-HCl buffer (pH 8.0). The active fractions were pooled and concentrated with Amicon filters to obtain the RgpA and Kgp enzymes.

Assay of gingipain activity

The enzymatic activity of gingipain, which is classified into cysteine proteases, was measured using two synthetic substrates, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA) and benzyloxy -carbonyl-L-histidyl-L-glutamyl-L-lysine 4-methylcoumaryl -7-amide (Z-His-Glu-Lys-MCA), in a 50 mM sodium acetate buffer (pH 6.5) containing 100 mM NaCl and 10 mM DTT. To a solution containing 50 μl of the given concentrations of inhibitor, we added an equal volume of purified RgpB (1 μg/ml) or RgpA-Kgp protease complex (3 μg/ml) in the same buffer and incubated for 15 min at 25℃. A reaction was started by the addition of 100 μl of 50-μM substrate incubated at 40℃ for 10 min, and the resulting released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 355 nm) on a fluorescence spectrophotometer.

RESULTS AND DISCUSSION

Genetic analysis of aflatoxin biosynthesis in *A. oryzae* S-03

The DNA target regions, which are widely used to identify *Aspergillus* species, are mainly internal transcribed spacer (ITS) regions in the rDNA complex (27). In this study, we carried out the direct sequencing of the PCR product of the S-03 DNA using ITS4 and ITS5 primers. The amplification of the ITS region of the strain generated a PCR product with 507 bp. The nucleotide sequence analysis showed that the S-03 strain is obviously classified as an A1a type of *A. oryzae-flavus* (data not shown).

Aflatoxin-biosynthesizing genes are still present in many strains of the genus *Aspergillus* (28~30). Since *A. oryzae* is genetically very close to the aflatoxin-producing *A. flavus*, there is a possibility that the S-03 strain has an aflatoxin-synthesizing gene cluster in its genome. Therefore, it is important to prove at the molecular level that *A. oryzae* S-03 is incapable of producing aflatoxin to continue using strains of this species with confidence in the food-processing industry. In previous research, an aflatoxin biosynthetic gene cluster was identified. The cluster includes a regulatory gene, designated *aflR*, and several structural genes, such as *nor-1*, *ver-1*, and *vbs* (31). To assess whether these genes are actively expressed in *A. oryzae* S-03 used in this study, a multiplex PCR was developed for the S-03 strain using seven sets of primer for *aflT*, *nor-1*, *aflR*, *norA*, *avnA*, *verB*, and *vbs* genes that cover sequences throughout the aflatoxin biosynthetic gene cluster. Instead, three amplification products corresponding to *avnA*, *verB*, and *vbs* genes were observed (Fig. 1), indicating that the S-03 strain belongs to the group 2 *Aspergillus*. Regarding the smear with lesser intensity which appeared in lane 2, we should note that this

Table 1. Primers used in this study

Name	Sequence	Tm
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	60.4
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	54.3
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG-3'	54.8
aflT(1)	5'-GCACCAAATGGGTCTTTCTCGT-3'	58.6
aflT(2)	5'-ATCCACGGTGAAGAGGGTAAGG-3'	60.4
nor1-A	5'-CGGACGAGGTCTCATTGAAGCTTT-3'	60.5
nor1-B	5'-ATCGATGATGAAGGCCGTGA-3'	56.3
new aflR(1)	5'-CCGGCGCATAACACGTACTC-3'	60.4
new aflR(2)	5'-GGCGCTTGGCCAATAGGTTC-3'	60.4
norA(1)	5'-GGCTGGAAAGGGGTAATGGG-3'	60.4
norA(2)	5'-TCTTGCGACCCTCACGAGAA-3'	58.4
avnA(1)	5'-AATCGCACCCAATGAGCTGTCT-3'	58.6
avnA(2)	5'-ATGGCCCGGGTTCTTTAGCAAC-3'	60.4
verB-A	5'-GATGCACCATGACCTCATGCGTTA-3'	60.5
verB-B	5'-CACGGCAGCGTTATTGATCATCTC-3'	60.4
vbs(1)	5'-TGCGAATGCTACGGCTCTCA-3'	58.4
vbs(2)	5'-CAACCGCCATCTCCTGGTCT-3'	60.4
no homology(1)	5'-CCTCGGAGACCGAAATATCTGAATACC-3'	62.0
no homology(2)	5'-CGGCGTACCAAATTCATTGCTTAC-3'	58.8
Tubulin(1)	5'-CCAAGAACATGATGGCTGCT-3'	56.3
Tubulin(2)	5'-CTTGAAGAGCTCCTGGATGG-3'	58.4

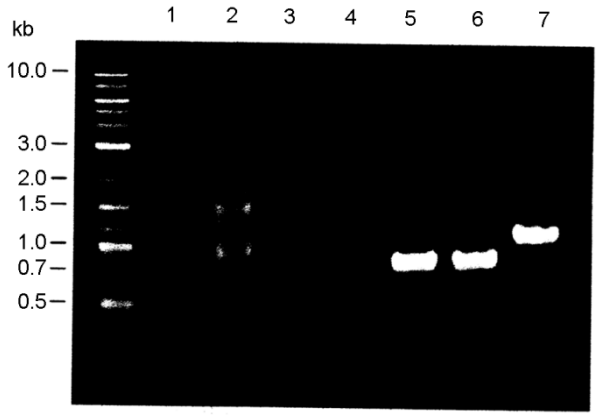

Figure 1. PCR amplification specificity of the genomic DNA of *A. oryzae* S-03 using primers designed for the aflatoxin gene cluster. The primer sequences are shown in Table 1. Lanes: 1, *aflT*; 2, nor-1; 3, *aflR*; 4, *norA*; 5, *avnA*; 6, *verB*; 7, *vbs*

smeared band is of inappropriate size for *nor-1* gene (529 bp). A further validation of S-03 strain classification, PCR amplification was carried out using Group 2 special primers, no-homology -F and -R and tubulin -1 and -2 listed in Table 1.

Furthermore, Southern blot analysis using the probe for the *aflR*-encoding region reveals that this gene is obviously absent in *A. oryzae* S-03 (Fig. 2). This result indicates that *A. oryzae* S-03 does not produce aflatoxin due to the lack of this major transcriptional regulator of the aflatoxin-biosynthetic genes and, therefore, is useful as a completely safe industrial strain. In fact, genomic analysis of the RIB40 strain as a typical *A. oryzae* has shown that deletions and the frame-shift mutations exist in the aflatoxin-encoding gene cluster. In addition, it is demonstrated that there are

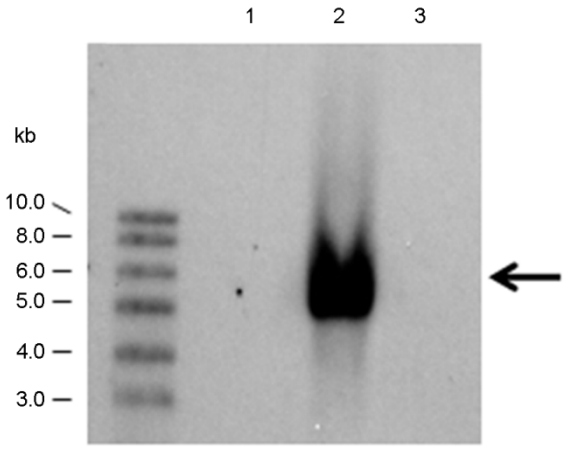

Figure 2. Southern blot analysis of the *Pst*I-digested chromosomal DNA from *Aspergillus* species. The *aflR* fragment was used as a probe. The arrow indicates the molecular mass of the signals. Lanes 1, *A. oryzae* M-01; 2, *A. oryzae* W-52; 3, *A. oryzae* S-03. The M-01 and W-52 strains are classified into group 2 and group 1, respectively. The *aflR* gene found in the W-52 strain, but not in the M-01 and S-03 strains.

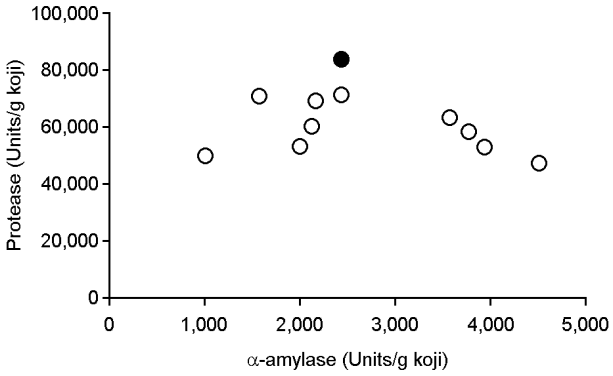

Figure 3. Activities of α-amylase and protease produced by *A. oryzae*. The detailed measurement method is described in the Materials and Methods section. Hatched rectangle, *A. oryzae* S-03.

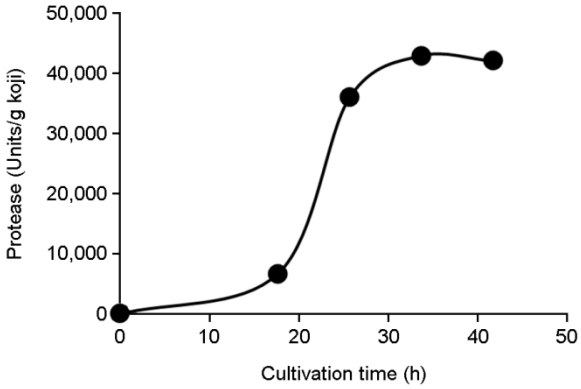

Figure 4. Effect of cultivation time on maximal protease production by *A. oryzae* S-03.

base pair substitutions on the chromosome as compared with the corresponding gene cluster in *A. flavus* (32, 33). It also has been reported that the deletion of the *aflR* gene in *A. nidulans* results in the lack of toxin-producing ability (34, 35).

Enzyme and antibacterial substance production by solid-state fermentation of *A. oryzae* S-03

Aspergillus species produce high amounts of extracellular proteases in submerged and solid-state cultures and, the secretion of proteases results in the degradation of many heterologous proteins. Consequently, we have chosen to focus on the fat-removed soybeans for the cultivation of *A. oryzae* S-03. Subsequently, amylase and protease activities were investigated in 11 strains of *A. oryzae* including *A. oryzae* S-03, which were used in the soy source production and provided from Higuchi Matsunosuke Shoten Co., Ltd.. As shown in Fig. 3, The S-03 strain produced the highest amount of protease and the total activity reached up to 87,600 U/g of dry cell weight, but the α-amylase production of the strain is average in the *A. oryzae* used in this study. In general, the productivities of protease and amylase of *A. oryzae* are known to be time dependently increased until 50 hr culture. Fig. 4 shows that protease production of *A. oryzae* S-03 on the fat-removed soybean was increased rapidly after 18 h of cultivation and maximal when cultured for 34 h.

A few reports provide insights into the roles of fungus-fermented products in bacterial infection. It has been shown that the ethanol extract of the fermented product from *Monascus* grown on rice grains displays antibacterial activities against *Bacillus subtilis*, *Staphylococcus aureus*, and *Salmonella typhimurium* (36, 37). Similar results have also been obtained in a study of *Rhizopus oryzae* fermented on soybeans and rice (24). However, despite these claims,

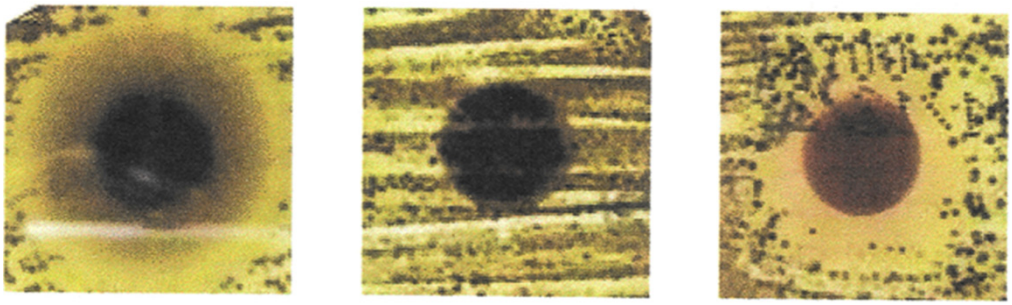

Figure 5. Antibacterial activity produced by *A. oryzae* S-03 cultivated on the fat-removed soybeans. The supernatant fluid was obtained extraction of koji and applied on paper disc. **1,** non-heat treated supernatant fluid; 2, the fluid treated with 100℃ for 10 min; 3, the fluid treated with 60℃ for 30 min.

Table 2. Activity of Rgp and Kgp proteases of *P. gingivalis* W83. The values are expressed as percentage of the activity determined in the absence of inhibitor.

Sample	Concentration (μg/ml)	Activity with non-treated samples (%)			Activity with heat treated samples (%)		
		RgpA-	Kgp	RgpB	RgpA	Kgp	RgpB
Control		100	100	100	100	100	100
LMWF extract	400	24.5	72.2	56.5	26.6	71.4	59.5
	200	65.4	91.2	74.4	81.3	92.0	75.7
	100	94.5	100	95.3	100	100	95.4

The purified RgpB or RgpA-Kgp protease complex were preincubated with the given concentrations of inhibitor for 15 min at 25℃.

there have been no scientific studies of *Aspergillus* species that describe the decrease or suppression of the toxicity of a pathogenic bacterium. To approach this issue, whether the fermented product with *A. oryzae* decreases or inhibits the function of virulence determinants of *P. gingivalis* was investigated. Our results have revealed that the supernatant fluid of fat-removed soybean koji cultivated with *A. oryzae* S-03 displays high antimicrobial activity against *P. gingivalis* (Fig. 5). The activity of the koji supernatant fluid was lost after heat treatment at 100℃ for 10 min. Further studies are now in progress to gain insight into the identification of the antibacterial substance and its molecular mechanisms that inhibit the cell growth of the bacterium.

Characterization of the *A. oryzae* S-03-produced inhibitor of gingipains

Since *P. gingivalis* is a highly proteolytic bacterium, it has been proposed that this property might contribute to the virulence of these organisms. Rgp and Kgp, which are cysteine-proteases secreted by the bacterium, function as major pathogens in the development and progression of periodontal diseases. Therefore, an inhibitor of the proteases would be an ideal target for preventing *P. gingivalis* colonization. The present study shows that an extract of the fat-removed soybean koji display significant inhibitory activity against both Kgp and Rgp. This inhibition characteristic was not significantly different throughout the fermentation process from 26 h to 42 h, a time interval coincident with the increase of the fungal protease rate in the molding process. Further, the extract was filtrated through an ultrafiltration membrane (MWCO, 3K) in order to fractionate gingipain inhibitory substances in high yield. High-molecular-weight retentive fractions scarcely arrested the gingipain activity, whereas low-molecular-weight per-

meable fractions (LMWFs) exhibited strong inhibitory effects against gingipains. The corresponding fraction on the fat-removed soybeans and the high-molecular-weight fraction from the extract of fat-removed soybean koji did not produce any compounds that against gingipain activity.

Table 2 shows that LMWFs inhibited low molecular weight RgpB activity by 25.6% and 43.5% at concentrations of 200 μg/ml and 400 μg/ml, respectively. With regard to the gingipain-adhesin complexes, the residual protease activity of RgpA, after exposure to 400 μg/ml LMWFs was 24.5%. The suppression was approximately 28% when proteolysis was induced by Kgp. It is generally thought that the Kgp gene product displays a 20% identity to the catalytic domain of RgpA, while the hemagglutinin domains are identical (Pavloff *et al*., 1995). If LMWFs are involved in the blocking of the hemagglutinin/adhesion domain of the bacterial protease, we would expect to see equal levels of inhibition regardless of the gingipain type. Thus, the inhibitory activity of LMWFs does not seem to be the result of blocking or interacting with the adhesin domain of the gingipain-adhesin complex. Furthermore, to determine whether heat treating LMWFs would alter the potency of their gingipain-inhibitory activities, the boiled sample was used for the protease assay. Table 2 shows that the activity of LMWFs against gingipains was not repressed, even after heat treatment at 100℃ for 15 min.

Soybeans have trypsin inhibitors, with molecular weights known to be 8 kDa and 20 kDa. Although we analyzed the inhibitory profile of LMWFs by SDS-PAGE (data not shown), no bands with the given molecular masses of 8 and 20 kDa were detected. We speculate that the gingipain inhibitor may be the digestive product of proteins contained in the fat-removed soybeans by the protease from *A. oryzae* S-03. Since koji contains phenolic compounds, the LMWF sample was also extracted 3 times with chloroform followed by 3 times with ethyl acetate. However, either with chloroform or with ethyl acetate, very little inhibitory activity against gingipains was observed as compared to that present on the residual water-soluble fraction (data not shown).

In conclusion, aflatoxin-biosynthesizing genes are still present in many strains of the genus *Aspergillus*. Therefore, it is important to prove at the molecular level that *A. oryzae* S-03 is incapable of producing aflatoxin to continue using strains of this species with confidence in the food-processing industry. By the nucleotide sequence and Southern blot analyses in this study, we have determined that the *aflR* necessary for regulating aflatoxin-biosynthetic genes is absent in *A. oryzae* S-03. Since the development of natural compounds with significant anti-bacterial targets has been the focus of much recent research, we further examined inhibitory activities against the oral pathogen of *P. gingivalis*. This bacterium produces several types of proteolytic enzymes, including gingipains, which are known to induce degradation of a broad range of connective tissues and host defense macromolecules. We show in the present study that *A. oryzae* S-03 exhibits both antibacterial and gingipain inhibitory activities when cultured onto the fat-removed soybeans. Of interest, gingipain inhibitory activities of the LMWF of the fat-removed soybean koji extract after heat treatment were practically indistinguishable.

REFERENCES

1) Abrar M, Anjum FM, Butt MS, Pasha I, Randhawa MA, Saeed F, *et al*. Aflatoxins: Biosynthesis, Occurrence, Toxicity and Remedies. Crit Rev Food Sci Nutr 2013; 53:862-74.
2) Hesseltine CW, Shotwell OL, Ellis JJ, Stubblefield RD. Aflatoxin formation by *Aspergillus flavus*. Bacteriol Rev 1966;30:795-805.
3) Kew MC. Aflatoxins as a cause of hepatocellular carcinoma. J Gastrointestin Liver Dis 2013;22:305-10.
4) Bhanja T, Kumari A, Banerjee R. Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. Bioresour Technol 2009;100:2861-6.
5) Hajjaj H, Duboc P, Fay LB, Zbinden I, Macé K, Niederberger P. *Aspergillus oryzae* produces compounds inhibiting cholesterol biosynthesis downstream of dihydrolanosterol. FEMS Microbiol Lett 2005;242:155 -9.
6) Inoue K, Gotou T, Kitajima H, Mizuno S, Nakazawa T, Yamamoto N. Release of antihypertensive peptides in

miso paste during its fermentation, by the addition of casein. J Biosci Bioeng 2009;108:111-5.
7) Gibson FC 3rd, Genco CA. *Porphyromonas gingivalis* mediated periodontal disease and atherosclerosis: disparate diseases with commonalities in pathogenesis through TLRs. Curr Pharm Des 2007;13:3665-75.
8) Griffen AL, Becker MR, Lyons SR, Moeschberger ML, Leys EJ. Prevalence of *Porphyromonas gingivalis* and periodontal health status. J Clin Microbiol 1998;36: 3239-42.
9) Chen T, Nakayama K, Belliveau L, Duncan MJ. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. Infect Immun 2001;69:3048-56.
10) Curtis MA, Aduse-Opoku J, Rangarajan M. Cysteine proteases of *Porphyromonas gingivalis*. Crit Rev Oral Biol Med 2001;12:192-216.
11) Imamura T. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol 2003;74:111-8.
12) Potempa J, Mikolajczyk-Pawlinska J, Brassell D, Nelson D, Thøgersen IB, Enghild JJ, *et al*. Comparative properties of two cysteine proteinases (Gingipains R), the products of two related but individual genes of *Porphyromonas gingivalis*. J Biol Chem 1998;273: 21648-57.
13) Potempa J, Sroka A, Imamura T, Travis J. Gingipains, the major cysteine proteinases and virulence factors of *Porphyromonas gingivalis*: structure, function and assembly of multidomain protein complexes. Curr Protein Pept Sci 2003;4:397-407.
14) Japoni A, Vasin A, Noushadi S, Kiany F, Japoni S, Alborzi A. Antibacterial susceptibility patterns of *Porphyromonas gingivalis* isolated from chronic periodontitis patients. Med Oral Patol Oral Cir Bucal 2011; 16:1031-5.
15) Kadowaki T, Baba A, Abe N, Takii R, Hashimoto M, Tsukuba T, *et al*. Suppression of pathogenicity of *Porphyromonas gingivalis* by newly developed gingipain inhibitors. Mol Pharmacol 2004;66:1599-606.
16) Bedi GS. Comparative study of four proteases from spent culture media of *Porphyromonas gingivalis* (FAY-19M-1). Prep Biochem 1995;25:133-54.
17) Kitano S, Irimura K, Sasaki T, Abe N, Baba A, Miyake Y, Katunuma N, *et al*. Suppression of gingival inflammation induced *Porphyromonas gingivalis* in rats by leupeptin. Jpn J Pharmacol 2001;85:84-91.
18) Monti B, Sparapani M, Contestabile A. Differential toxicity of protease inhibitors in cultures of cerebellar granule neurons. Exp Neurol 1998;153:335-41.
19) Labrecque J, Bodet C, Chandad F, Grenier D. Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of *Porphyromonas gingivalis*. J Antimicrob Chemother 2006;58:439-43.
20) Okamoto M, Sugimoto A, Leung KP, Nakayama K, Kamaguchi A, Maeda N. Inhibitory effect of green tea catechins on cysteine proteinases in *Porphyromonas gingivalis*. Oral Microbiol Immunol 2004;19:118-20.
21) Sakanaka S, Aizawa M, Kim M, Yamamoto T. Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, *Porphyromonas gingivalis*. Biosci Biotechnol Biochem 1996;60:745-9.
22) Toh EC, Dashper SG, Huq NL, Attard TJ, O'Brien-Simpson NM, Chen YY, *et al*. *Porphyromonas gingivalis* cysteine proteinase inhibition by κ-casein peptides. Antimicrob Agents Chemother 2011;55:1155 -61.
23) Miyasaki KT, Iofel R, Oren A, Huynh T, Lehrer RI. Killing of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* by protegrins. J Periodontal Res 1998;33:91-8.
24) Wang HL, Ellis JJ, Hesseltine CW. Antibacterial activity produced by molds commonly used in oriental food fermentations. Mycologia 1972;64:218-21.
25) Jin J, Lee YK, Wickes BL. Simple chemical extraction method for DNA isolation from *Aspergillus fumigatus* and other *Aspergillus* species. J Clin Microbiol 2004; 42:4293-6.
26) Potempa J, Nguyen KA. Purification and characterization of gingipains. Curr Protoc Protein Sci 2007.
27) Henry T, Iwen PC, Hinrichs SH. Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. J Clin Microbiol 2000;38:1510-5.
28) Criseo G, Bagnara A, Bisignano G. Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. Lett Appl Microbiol 2001;33: 291-5.
29) El-Hag N, Morse RE. Aflatoxin production by a variant of *Aspergillus oryzae* (NRRL strain 1988) on cowpeas (Vigna sinensis). Science 1976;192:1345-6.

30) Tominaga M, Lee YH, Hayashi R, Suzuki Y, Yamada O, Sakamoto K, *et al*. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* RIB strains. Appl Environ Microbiol 2006;72:484-90.
31) Ehrlich KC, Yu J, Cotty PJ. Aflatoxin biosynthesis gene clusters and flanking regions. J Appl Microbiol 2005; 99:518-27.
32) Kiyota T, Hamada R, Sakamoto K, Iwashita K, Yamada O, Mikami S. Aflatoxin non-productivity of *Aspergillus oryzae* caused by loss of function in the *aflJ* gene product. J Biosci Bioeng 2011;111:512-7.
33) Machida M, Yamada O, Gomi K. Genomics of *Aspergillus oryzae*: learning from the history of Koji mold and exploration of its future. DNA Res 2008;15: 173-83.
34) Payne GA, Nystrom GJ, Bhatnagar D, Cleveland TE, Woloshuk CP. Cloning of the afl-2 gene involved in aflatoxin biosynthesis from *Aspergillus flavus*. Appl Environ Microbiol 1993;59:156-62.
35) Yu JH, Butchko RA, Fernandes M, Keller NP, Leonard TJ, Adams TH. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. Curr Genet 1996;29:549-55.
36) Kim EY, Rhyu MR. Antimicrobial activities of *Monascus koji* extracts. Korean J Food Sci Technol 2008;40:76-81.
37) Ungureanu C, Ferdes M. Antibacterial and antifungal activity of red rice obtained from *Monascus purpureus*. Chem Eng 2010;20:223-8.