

ORIGINAL ARTICLE

Inhibitory activity of blasticidin A, a strong aflatoxin production inhibitor, on protein synthesis of yeast: selective inhibition of aflatoxin production by protein synthesis inhibitors

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Blasticidin A (BcA), an antibiotic produced by *Streptomyces*, inhibits aflatoxin production without strong growth inhibition toward aflatoxin-producing fungi. During the course of our study on the mode of action of BcA by two-dimensional differential gel electrophoresis (2D-DIGE), we found a decrease in the abundances of ribosomal proteins in *Saccharomyces cerevisiae* after exposure to BcA. This phenomenon was also observed by treatment with blasticidin S (BcS) or cycloheximide. BcA inhibited protein synthesis in a galactose-induced expression system in *S. cerevisiae* similar to BcS and cycloheximide. BcS, but not cycloheximide, inhibited aflatoxin production in *Aspergillus parasiticus* without inhibition of fungal growth, similar to BcA. A decrease in the abundances of aflatoxin biosynthetic enzymes was observed in 2D-DIGE experiments with *Aspergillus flavus* after exposure to BcA or BcS. These results suggested that protein synthesis inhibitors are useful to control aflatoxin production.

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INTRODUCTION

Aflatoxins are a group of mycotoxins with potent toxicity and carcinogenicity in mammals. Some strains of *Aspergillus* sp. produce aflatoxins that are found as contaminants in a wide variety of food and feed commodities. Aflatoxin contamination in agricultural products is a serious problem in terms of not only food safety but also economic loss.^{1,2} However, it is difficult to resolve the problem due to lack of an effective method to control aflatoxin production.

We have been studying specific inhibitors of aflatoxin production based on the idea that they may be useful to prevent foods and feeds from aflatoxin contamination without incurring a rapid spread of resistant strains.³ Blasticidin A (BcA) was found as an antibiotic produced by *Streptomyces griseochromogenes*, but its structure had not been known.^{4,5} We found that BcA strongly inhibited production of both aflatoxin B and G groups by *Aspergillus parasiticus*, and we determined its structure,^{6,7} which is similar to that of aflastatin A, an aflatoxin production inhibitor produced by *Streptomyces* sp.⁸ BcA and aflastatin A reduced the mRNA levels of genes encoding aflatoxin biosynthetic enzymes and a key regulatory protein, AflR.^{9,10} Therefore, they may affect a pathway leading to expression of aflatoxin biosynthetic enzymes.

BcA inhibited aflatoxin production of *A. parasiticus* without significantly disturbing the fungal growth at low concentrations, but

inhibition of the fungal growth was observed at higher concentrations. This suggested that aflatoxin production inhibitory activity of BcA might be correlated with its antifungal activity. BcA showed antimicrobial activity toward *Saccharomyces cerevisiae*.¹⁰ Our attempt to obtain BcA-resistant strains by transformation of *S. cerevisiae* with cDNA or genomic libraries has not afforded critical candidate genes for investigating a target molecule (data not shown). Therefore, analysis of the yeast proteome by two-dimensional differential gel electrophoresis (2D-DIGE)¹¹ was performed. There are many works on proteome of *S. cerevisiae*¹² and alterations in protein abundance levels can provide clues about the mechanism of action of various drugs.¹³ However, it is still difficult to estimate the target molecule of a drug from proteome information alone. In this study, we found relationship between protein synthesis inhibition and decrease of abundances of ribosomal proteins in *S. cerevisiae*. We also performed 2D-DIGE analysis with *Aspergillus flavus* and showed decrease of abundances of aflatoxin biosynthetic enzymes by treatment with some aflatoxin production inhibitors.

In this paper, we report inhibitory activity of BcA on protein synthesis of *S. cerevisiae* and selective inhibition of aflatoxin production of *Aspergillus* sp. by some protein synthesis inhibitors.

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MATERIALS AND METHODS

Strains, growth media and chemicals

S. cerevisiae strain W303, *A. parasiticus* NRRL2999 and *A. flavus* IMF47798 were used. Details of culture conditions are described in Supplementary information. Analysis of growth and aflatoxin production was performed as described earlier.¹⁴

BcA (purified from the culture broth of *S. griseochromogenes* IFO13413, purity >98% determined by HPLC), rapamycin (Sigma-Aldrich, St Louis, MO, USA) and amphotericin B (Wako, Osaka, Japan) were dissolved in dimethyl sulfoxide. Cycloheximide (Nacalai tesque, Kyoto, Japan), G418 (Invitrogen, Carlsbad, CA, USA) and blasticidin S (BcS) (Invitrogen) were dissolved in distilled water.

Sample preparation for 2D-DIGE analysis

S. cerevisiae cells were cultured overnight and diluted to an OD₆₀₀ of 0.4, and then cultured for 1 h. A test drug was added to the cultures, followed by 30 min incubation. In the case of *A. flavus*, a spore suspension of the fungus (10 µl) was added to a Potato Dextrose (Becton, Dickinson and Company, Sparks, MD, USA) medium (10 ml) with BcA (0.3 µM) or BcS (300 µM) in a 100 ml Erlenmeyer flask and incubated. After 24 or 36 h, mycelia were harvested and lyophilized. Total proteins of the yeast cells or fungal mycelia were extracted using CelLytic Y Yeast Cell Lysis/Extraction Reagent (Sigma-Aldrich) with protease inhibitor cocktail EDTA-free (Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein samples were precipitated with acetone and treated with 2D-DIGE lysis buffer (2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 30 mM Tris-HCl, pH 8.5). Total protein contents were determined by the Bradford assay using BSA as a standard.

Fluorescence labeling with Cy-Dyes

Drug-treated and control samples were quantified and labeled with NHS-Cy2, -Cy3 and Cy5 (GE Healthcare, Buckinghamshire, UK). Protein (50 µg) taken from drug-treated or control samples were minimally labeled with 160 pmol of Cy3 or Cy5 in triplicate. An equal pool of drug-treated and control samples were labeled with Cy2 (3.2 pmol µg⁻¹ protein) and run as a standard on all gels to aid in spot matching and cross-gel quantitative analysis. Protein labeling was performed on ice in the dark for 30 min. Reactions were quenched by 10 min incubation with a 20-fold molar excess of free lysine. The labeled samples were mixed and carrier Pharmalyte (pH 3–10, GE Healthcare) was added to a final concentration of 2%.

2D-gel electrophoresis

Control, drug-treated and internal standard samples labeled with Cy3, Cy5 and Cy2, respectively, were mixed and separated by isoelectric focusing in the first dimension using 24 cm pH 3–10 NL (nonlinear) or 3–5.6 NL IPG strip (GE Healthcare) and by SDS-PAGE in the second dimension using 10% SDS-PAGE gel bonded to low-fluorescence glass plates. Gels were run in Ettan 6 (GE Healthcare) gel tanks at 400 mA per gel at 20 °C until the dye front had run off the bottom.

Image acquisition and biological variance analysis

After 2D-gel electrophoresis, gels were scanned using a Typhoon 9400 variable mode imager (GE Healthcare) and ImageQuant software (GE Healthcare). The photomultiplier tube voltage was adjusted for each dye channel for preliminary low-resolution scans to give maximum pixel values within 5–10% for each channel and below saturation, before the acquisition of 100 µm high-resolution images. Images were cropped and analyzed using DeCyder V5.0 (GE Healthcare). Ratios of spot intensities detected in drug-treated or control samples to those of corresponding spots in standard sample were calibrated and obtained values were averaged across triplicates for each experimental condition. Statistical analysis was performed to pick spots matching across all images, displaying a ≥ 1.4 (in experiments with *S. cerevisiae*) or 1.5 (in experiments with *A. flavus*) average-fold increase or decrease in abundance between drug-treated and control samples and with *P*-values <0.05 (Student's *t*-test).

In-gel digestion and protein identification by mass spectrometry

Visible spots were excised from the CBB R250 (Nacalai tesque)-stained gels and transferred into microcentrifuge tubes. Excised fragments were washed successively with water, 25 mM NH₄HCO₃, acetonitrile per 25 mM NH₄HCO₃ (1:1) and acetonitrile. Destained gel fragments were dried under vacuum with a centrifugal evaporator. Tryptic digestion was performed overnight at 37 °C using 10 µl of 10 µg ml⁻¹ trypsin (Roche) in 50 mM NH₄HCO₃, pH 7.8. The resulting tryptic fragments were extracted twice with 100 µl of acetonitrile/water (3:2) containing 0.1% TFA, in an ultrasonic bath for 15 min. The supernatants were concentrated to 10 µl in a centrifugal evaporator and subjected to LC-MS/MS analysis. The concentrated solutions were passed through a Zip-Tip C18 (Millipore, Bedford, MA, USA). The adsorbed peptides were eluted with 2.0 µl of acetonitrile/water (3:2) containing 0.1% TFA and 1% α -cyano-4-hydroxycinnamic acid. The eluent was loaded onto a mass spectrometer sample plate. Mass spectra were acquired on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated in the reflectron-delayed extraction mode. Spectra were internally calibrated using trypsin autodigestion products. MASCOT (Matrix Science, Boston, MA, USA) was used to search databases. The expect value, number of matched peptides and percentage of sequence coverage for each identified protein are listed in Tables 1 and 2. *A. flavus* proteins in spots 6, 8 and 12 (Supplementary Figure S2; Table 2) were identified by LC-MS/MS analysis according to the methods in Supplementary information.

Protein synthesis inhibitory activity of BcA

Strain w303 was freshly transformed with plasmid pYES2-6 \times His-LacZ (Invitrogen) and grown overnight in SD-ura raffinose media. This culture was diluted to an OD₆₀₀ of 1.0. A test compound and 600 µl of SD-ura galactose media were added to 2.4 ml of the diluted culture. The cells were incubated for 60 min and harvested, and total protein extractions were performed as described above. After acetone precipitation, the extracted proteins were re-suspended in SDS-PAGE sample buffer (65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue). Proteins were separated on 7.5% SDS-PAGE gels and transferred to a PVDF membrane. The membrane was probed using an anti-6 \times His-tag antibody (BioDynamics Laboratory, Tokyo, Japan) and visualized by using immobilon Western Chemiluminescent HRP Substrate (Millipore).

RESULTS AND DISCUSSION

Effects of BcA on *S. cerevisiae* proteome

S. cerevisiae was incubated without or with BcA (1 µM corresponding to MIC) for 30 min and the proteins were extracted from the cells. The control and BcA samples were labeled with green and red dyes, respectively, and combined. The mixture was separated by 2D-gel electrophoresis followed by fluorescent imaging (Figure 1). Five green (spots 1, 2, 3, 4 and 5) and one red (spot 6) spots, showing changes in abundance >1.4-fold, were clearly observed by using the gel with a pH range of 3–10 (Figure 1a). A better separation of spots 7, 8, 9 and 10 was obtained using a pH range of 3–5.6 (Figure 1b). Proteins in spots 1, 2, 3, 4 and 5 (decreased with BcA treatment) and spot 6 (increased with BcA treatment) were identified by MALDI-TOF MS analysis after in-gel tryptic digestion as ribosomal proteins (Rpp0p, Rps0Ap, Rps0Bp, Asc1p and Rps5p) and glycerol-3-phosphate dehydrogenase (Gpd1p) (Table 1). Results of the MASCOT search analysis on proteins in spots 7a, 8a, 9a and 10a showed that they were the same as those in spots 7b, 8b, 9b and 10b, respectively. The proteins were identified as translational factors (Sup45p and Tif5p) and molecular chaperones (Pdr13p and Cdc37p) (Table 1).

In all these paired spots, increase of the levels of protein molecules with lower isoelectronic points and concomitant decrease of those with higher ones were observed. MS analysis of spot 8b yielded an (M+H)⁺ ion peak for a trypsinized peptide corresponding to amino-acid residues 383–405 of Tif5p at *m/z* 2682.43 (Supplementary Figure S1a). In contrast, MS analysis of spot 8a yielded an ion peak

Table 1 Analysis of effects of protein synthesis inhibitors by 2D-DIGE

Spot	Protein	Molecular function ^b	Fold change ^a										Matched peptides	Sequence coverage (%)
			Blasticidin A ^c	Blasticidin S ^d	Cycloheximide ^e	G418 ^f	Rapamycin ^g	Amphotericin B ^h	pI ^b	MW (k) ^b	Expect			
1	RppOp	Ribosomal protein PO	-1.52	-1.76	-1.51	+1.01	+1.06	+1.03	4.75	33.7	0.00015	5	21	
2	Rps0Ap	Ribosomal protein of the small subunit	-1.48	-1.26	-1.33	+1.01	+1.03	+1.17	4.65	27.9	4.30E-12	8	39	
3	Rps0Bp	Ribosomal protein of the small subunit	-1.54	-1.60	-1.51	-1.03	-1.10	+1.18	4.69	27.8	1.70E-12	8	42	
4	Asc1p	Core component of the small ribosomal subunit	-1.81	-1.77	-1.65	-1.15	+1.02	+1.01	5.80	34.7	5.40E-16	10	54	
5	Rps5p	Ribosomal protein of the small subunit	-1.41	-1.47	-1.35	-1.00	+1.09	+1.17	8.62	25.0	1.00E-05	5	19	
6	Gpd1p	Glycerol-3-phosphate dehydrogenase	+2.10	+1.05	+1.07	-1.02	-1.02	+1.03	5.30	42.7	2.20E-05	5	17	
7a	Sup45p acid	Polypeptide release factor	+1.67	+1.02	+1.04	-1.05	+1.07	+1.08	4.94	49.0	8.90E-05	5	13	
7b	Sup45p base	Polypeptide release factor	-2.05	+1.06	+1.13	-1.01	-1.03	+1.01	4.94	49.0	0.0042	4	10	
8a	Tif5p acid	Translation initiation factor	+1.39	ND ^h	ND	ND	ND	ND	4.95	45.3	1.70E-06	6	18	
8b	Tif5p base	Translation initiation factor	-3.46	ND	ND	ND	ND	ND	4.95	45.3	4.30E-11	9	25	
9a	Pdr13p acid	Hsp70 protein that functions as part	+1.49	ND	ND	ND	ND	ND	4.94	58.2	2.10E-14	11	24	
9b	Pdr13p base	of the ribosome-associated complex	-1.11	ND	ND	ND	ND	ND	4.94	58.2	1.40E-09	8	18	
10a	Cdc37p acid	Hsp90p co-chaperone	+2.65	ND	ND	ND	ND	ND	4.94	58.4	0.48	3	4	
10b	Cdc37p base	Hsp90p co-chaperone	-1.77	ND	ND	ND	ND	ND	4.94	58.4	0.48	3	4	

Abbreviation: 2D-DIGE, two-dimensional differential gel electrophoresis.

^aNormalized spot intensity: drug treated vs untreated cells (average of triplicate gels from each of the three independent cultures).

^bProtein molecular function, theoretical pI (isoelectric point) and MW (molecular weight) were obtained from SGD (<http://www.yeastgenome.org>).

^c1 μM.

^d300 μM.

^e3 μM.

^f600 μM.

^gRapamycin (0.2 μM) and amphotericin B (1 μM) were used as control drugs with a known mode of action.

^hNot detected.

Table 2 Identification of protein spots in 2D-DIGE experiments with blasticidin A and blasticidin S treated *A. flavus* cells

Spot	NCBI locus	Molecular function	Fold change ^a						Matched peptides	Sequence coverage (%)
			Blasticidin A		Blasticidin S		MW (k)	Expect		
			24 h	36 h	36 h	pI				
1	AAS90106	VBS	ND ^b	-1.75	-2.79	5.17	70.3	1.60E-09	9	14
2	AAS90080	OmtB	ND	-3.07	-3.52	5.22	43.3	0.02	4	12
3	AAS90104	OmtA	ND	-2.05	-2.00	5.80	46.8	3.10E-09	8	23
4	AAS90074	NorA	ND	-4.06	-3.10	6.28	43.8	0.00037	5	13
5	AAS90030	Ver-1	ND	-4.60	-6.07	6.32	27.9	2.00E-17	12	35
6	AAS90070	Nor-1 ^c	ND	ND	-2.90	6.79	29.4	—	—	—
7	BAE63671	Predicted protein	-1.72	-1.61	-2.24	4.72	52.5	0.001	5	11
8	BAE65896	Conserved hypothetical protein ^c	-1.70	-1.89	-4.94	6.94	82.6	—	—	—
9	BAE62700	Catalase B	-1.45	-1.69	-2.73	5.34	79.8	4.00E-07	8	11
10	BAE62196	Copper amine oxidase	-1.43	-1.58	-1.55	5.95	75.2	1.60E-17	14	35
11	BAE63124	Chitosanase	ND	-1.63	-3.44	4.81	39.5	0.75	3	10
12	CAK39058	Short chain dehydrogenase/ reductase family oxidoreductase, putative ^c	-1.64	-1.18	-2.59	5.20	25.2	—	—	—
13	BAE59504	Amine oxidase, flavin-containing superfamily	ND	+1.09	-3.18	4.83	50.8	1.9	3	7
14	BAE59174	Catalase	-1.07	-1.15	-1.63	8.34	40.0	6.30E-06	6	21
15	BAE56828	Conserved hypothetical protein	ND	-1.17	-2.47	5.44	38.5	3.10E-13	10	18
16	BAE58779	ATP-citrate lyase	-1.10	-1.07	-1.50	7.62	68.6	7.90E-08	8	19
17	BAE59009	Aldehyde dehydrogenase	-1.31	-1.13	-1.90	5.84	57.6	1.60E-15	12	38
18	ABP52074	Glutathione S-transferase κ subfamily	+1.63	+1.90	+1.62	5.10	24.6	1.00E-08	7	29
19	BAE56408	Glutathione S-transferase	+3.63	+4.59	+1.57	5.53	29.4	1.30E-08	7	27
20	BAE66068	Conserved hypothetical protein	ND	+2.40	ND	5.55	20.2	1.60E-07	6	24
21	BAE56464	Pyruvate kinase	+1.18	+1.22	+1.56	6.41	58.1	5.00E-07	7	23

Abbreviation: 2D-DIGE, two-dimensional differential gel electrophoresis.

^aNormalized spot intensity: blasticidin A or blasticidin S treated vs untreated cells (average of triplicate gels from each of the three independent cultures).

^bNot detected.

^cIdentified by amino-acid sequences from LC-MS/MS analysis.

at m/z 2762.72 instead of m/z 2682.43 (Supplementary Figure S1b). Ser-397 of Tif5p is a possible phosphorylation site. These results suggested that the acidic nature of Tif5p in spot 8a was due to phosphorylation and that BcA may promote phosphorylation of Tif5p. Phosphorylation of Tif5p is catalyzed by protein kinase CK2.¹⁵ CK2 phosphorylates a variety of substrate proteins including Sup45p and Cdc37p.¹⁶ Therefore, phosphorylation of Sup45p and Cdc37p may be promoted by BcA when BcA activates CK2.

Effects of BcA on gene transcription

Quantitative PCR analysis was performed to examine the effect of BcA on the transcription of genes encoding the proteins whose abundance levels were altered by BcA treatment. *S. cerevisiae* cells were treated with BcA (1 μM) or rapamycin (0.2 μM corresponding to MIC). Rapamycin reduces transcription of genes encoding ribosomal proteins.¹⁷ The mRNA levels of ribosomal protein genes, *ASC1* and *RPP0*, were reduced by rapamycin, but were not affected by BcA (Figures 2a and b), indicating that BcA decreased abundance levels of these ribosomal proteins without affecting transcription. On the other hand, the mRNA level of *GPD1* was clearly increased by BcA (Figure 2c).

Gpd1p is an enzyme required for glycerol synthesis and mRNA level of *GPD1* is upregulated by high osmotic stress.¹⁸ Phosphorylation of Cdc37p is important in the high osmotic stress signal transduction pathway that leads to glycerol synthesis.¹⁹ Therefore, increase of levels of Gpd1p and phosphorylated Cdc37p by BcA treatment suggested

that BcA might affect the high osmotic stress signal transduction pathway by increasing Cdc37p phosphorylation.

Effects of BcA on protein synthesis in *S. cerevisiae*

The protein synthesis inhibitory activity of BcA was examined as most proteins identified in the 2D-DIGE experiments were involved in protein translation. *S. cerevisiae* was transformed with a plasmid containing the *GAL1* promoter and histidine-tagged β-galactosidase (6×His-LacZ) gene. The transformed cells did not produce 6×His-LacZ protein in a medium containing glucose or raffinose due to glucose repression of the *GAL1* promoter (Figure 3, lane A). Induction of 6×His-LacZ protein synthesis was observed when cells having been cultured in a raffinose medium were inoculated into a medium with galactose (lane B). In this galactose-induction protein synthesis system, eukaryotic protein synthesis inhibitors, cycloheximide and BcS, clearly inhibited synthesis of 6×His-LacZ protein (lanes F and H), but amphotericin B had no effect (lane G). Similarly to cycloheximide and BcS, BcA inhibited 6×His-LacZ protein synthesis dose dependently (lanes C, D and E). This strongly suggested that BcA has inhibitory activity toward protein synthesis in *S. cerevisiae*.

Effects of protein synthesis inhibitors on *S. cerevisiae* proteome

Next, we examined the effect of known protein synthesis inhibitors on the *S. cerevisiae* proteome. *S. cerevisiae* cells were treated with the MIC concentration of three inhibitors effective in eukaryote, cycloheximide, BcS and G418, and rapamycin for 30 min. Cycloheximide and BcS

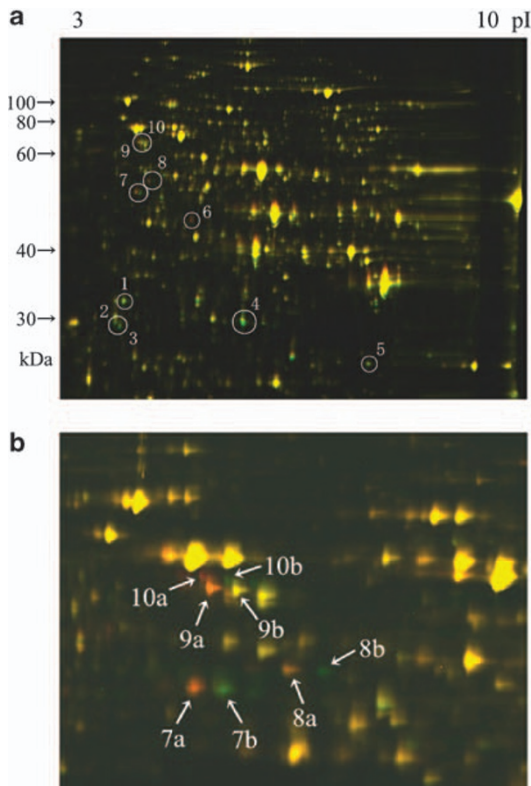


Figure 1 2D-DIGE gel image of *S. cerevisiae* proteome after blasticidin A treatment. (a) pI range 3–10. (b) pI range 3–5.6. Green, red and yellow spots indicate proteins with decreased, increased or unchanged abundance levels, respectively. Selected proteins (>1.4 and <-1.4 change in abundance; $P<0.05$; $n=3$) are numbered.

decreased abundances of the same five ribosomal proteins as BcA (Table 1). The speed of synthesis of ribosomal proteins is known to be fastest among all proteins in yeast.²⁰ Therefore, reduction of ribosomal proteins might be observed more remarkably than others when protein synthesis is inhibited. Rapamycin and G418 did not affect abundance levels of the ribosomal proteins including Asc1p and Rpp0p with the short time treatment conditions used in this study. Abundance levels of Sup45p and Gpd1p were not affected by cycloheximide, BcS, G418 or rapamycin, but were affected by BcA mentioned above. Tif5p, Sup45p and Pdr13p²¹ are important factors for translation in *S. cerevisiae*, but phosphorylation of Tif5p and Sup45p²² is not essential for ribosomal function of the yeast and there is no information on phosphorylation of Pdr13p. Therefore, it is unclear whether increased levels of phosphorylated Tif5p, Sup45p or Pdr13p resulting from BcA treatment is correlated with protein synthesis inhibition.

Aflatoxin production inhibitory activity of protein synthesis inhibitors

The effects of protein synthesis inhibitors on aflatoxin production were examined. *A. parasiticus* was cultured in a liquid medium containing a test compound. The weight of fungal mycelia and aflatoxin content in the culture broth were measured (Table 3). The ratio of IC₅₀ for growth inhibition based on mycelial weight to aflatoxin production inhibition was used as an indicator of specificity for aflatoxin production inhibition (shown as g/a values in Table 3). BcA was the strongest inhibitor of aflatoxin production with relatively high selectivity (g/a 6.4). BcS and G418 were also shown to be specific

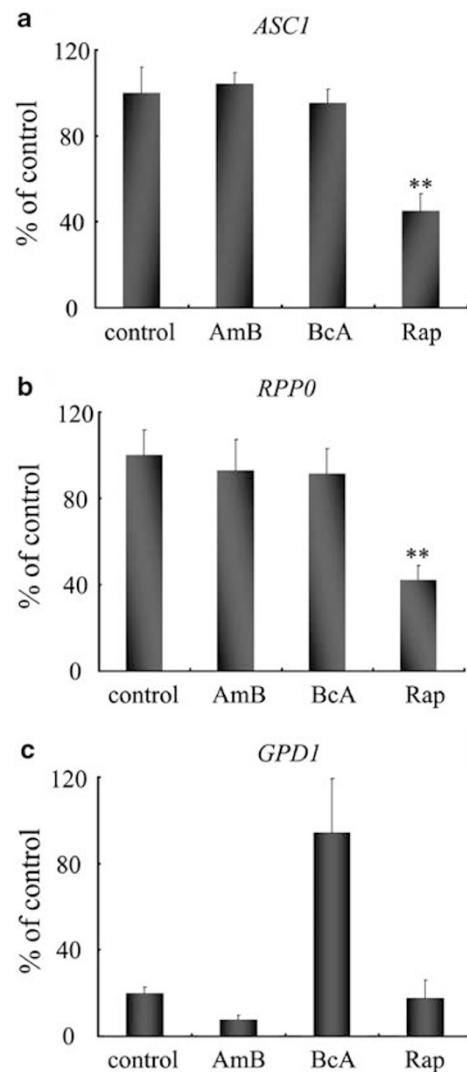


Figure 2 Effects of blasticidin A on transcription of *ASC1* (a), *RPP0* (b) and *GPD1* (c). Total RNA was prepared from yeast that had been cultured in a YPD liquid medium with or without BcA (1 μ M), amphotericin B (AmB, 1 μ M) or rapamycin (Rap, 0.2 μ M) for 30 min. AmB was used as a control drug with a known mode of action. The amount of each mRNA was normalized by the amount of β -actin mRNA in each sample. Data are presented as the mean \pm s.d. ($n=3$). ** $P<0.01$, vs control (one-way analysis of variance, followed by Dunnett test).

aflatoxin production inhibitors (g/a >36 and 8.0, respectively). Especially, BcS had very high selectivity. Cycloheximide inhibited both mycelial growth and aflatoxin production without selectivity (g/a 1.4). Chloramphenicol, neomycin and streptomycin, which are effective only in prokaryote, did not influence aflatoxin production or fungal growth.

Effects of BcA and BcS on *A. flavus* proteome

Proteome experiments with *A. flavus*, whose genome sequence has been clarified, were done next. BcA and BcS inhibited aflatoxin production of *A. flavus* (IC₅₀ 0.25 and 28 μ M, respectively) with selectivity (IC₅₀ for growth 1.6 μ M and >1000 μ M, respectively), similarly to the case of *A. parasiticus*. *A. flavus* was cultured with BcA (0.3 μ M) or BcS (300 μ M) for 24 h (before onset of aflatoxin production) or 36 h (after onset of aflatoxin production), and proteins

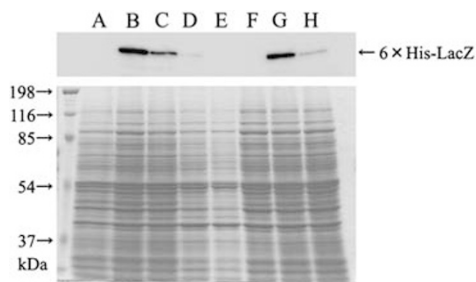


Figure 3 Inhibition of *S. cerevisiae* protein synthesis in the galactose-induction system. Cell extracts were analyzed by immunoblotting with anti-6×His antibody (upper panel) or SDS-PAGE after CBB staining (lower panel). Lane A: no galactose (negative control); lane B: no drug (positive control); lane C: 0.1 μM blasticidin A; lane D: 0.3 μM blasticidin A; lane E: 1 μM blasticidin A; lane F: 3 μM cycloheximide; lane G: 1 μM amphotericin B; lane H: 300 μM blasticidin S.

Table 3 Inhibitory activity of protein synthesis inhibitors against growth and aflatoxin production of *A. parasiticus*

Compound	$IC_{50}(\mu\text{M})$		
	Aflatoxin ^a production (a)	Growth (g)	g/a^b
Blasticidin A	0.25	1.6	6.4
Blasticidin S	28	>1000	>36
G418	20	160	8.0
Cycloheximide	26	36	1.4
Chloramphenicol	>3000	>3000	
Neomycin	>1600	>1600	
Streptomycin	>1700	>1700	

^aThis aflatoxin means total of aflatoxin B₁ and G₁, which are the major detectable aflatoxins produced by the fungus.

^bThe value of g/a means IC_{50} value for growth (g)/ IC_{50} value for aflatoxin production (a).

extracted from the mycelia were analyzed by 2D-DIGE (Supplementary Figure S2). We identified 5, 13 and 20 proteins whose abundance levels were changed after 24 h BcA treatment, 36 h BcA treatment and 36 h BcS treatment, respectively (Table 2). Decreased abundances of aflatoxin biosynthetic enzymes (Vbs, OmtB, OmtA, NorA, Ver-1, Nor-1) were observed after 36 h treatment with BcA or BcS. Abundance levels of proteins in spots 7–11 (Table 2) were also decreased with both BcA and BcS treatment. Significant decreased levels of proteins in spots 7 and 8 were also observed with 24 h BcA treatment. Functions of most of these proteins are unclear, but they and proteins obtained by further proteome analysis might afford a clue to investigate the regulatory mechanism for aflatoxin production.

Our results strongly suggest that protein synthesis inhibition is a key mode of action of BcA for its antimicrobial activity toward the yeast. It is not clear whether protein synthesis inhibitory activity of BcA causes selective inhibition of aflatoxin production in fungi, but we found the selective inhibition of BcS and G418 toward the aflatoxin

production. Toxicity of BcS has been examined thoroughly and BcA had been practically used in agriculture as an anti-rice blight drug for more than last four decades. Therefore, BcS may be a candidate or lead compound as an effective drug for preventing foods and feeds from aflatoxin contamination. Although it is unclear whether the different effects of BcS, G418 and cycloheximide on aflatoxin production were due to their different modes of inhibition of protein synthesis, our results clearly showed that protein synthesis is a possible target for developing useful aflatoxin production inhibitors.

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