Effects of Cycasin on Various Microorganisms

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Introduction

From of old, seeds of the Japanese cycad, *Cycas revoluta* Thunb., have been known to be poisonous to humans and animals, and inadequate removal of toxin to bring about human poisoning. In the south-western islands of Japan, however, cycad seeds were utilized as a starchy food in famine years and as raw materials of bean paste, "Miso". Nowadays, "Miso" is rarely made from cycad seeds.

A toxic component named cycasin was isolated and identified as methylazoxymethyl-β-D-glucoside in our laboratory[23]. After cycasin was reported to be carcinogenic[18,29], there were some people feeling uneasy about cycad foods. Kobayashi, one of the present authors, and his co-workers proved that several samples of home-made "Sotetsu Miso (cycad bean paste)" collected on the island of Amami-Ohshima were free from cycasin[14,15]. In addition, Kobayashi *et al.* experimentally made "Sotetsu Miso" to clarify the decomposition-aspects of cycasin by koji mold during the process[16]. In their work, cycasin was shown to suppress the growth of koji mold.

We have examined the effects of cycasin on organisms other than animals[17]. In the previous paper, we reported the toxic effects of cycasin on plant seedlings[30]. This paper describes the effects of cycasin on various microorganisms including koji mold.

Materials and Methods

Reagents

DL- Glyceraldehyde-3-phopshate, glucose-6-phosphate, glucose-1-phosphate, peroxidase (horseradish, Type I) and glycerol-3-phosphate dehydrogenase (NAD$^+$) were purchased from Sigma Chemicals. Glucomesser reagent was obtained from Shinotesto Co. NADH and NADP$^+$ were products of Seikagaku Kogyo Co. D-Lactate dehydrogenase was obtained from Boehringer Mannheim Co., Sephadex G-25 from Pharmacia Fine Chemicals, kojic acid from Tokyo Chemical Industry, and other chemicals were from Nakarai Chemicals Co.

Cycasin was isolated from seeds of the Japanese cycad in our laboratory. *o*-Nitrophenyl-β-D-glucoside was prepared according to the method of Glaser and Wulwek[8].

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Microorganisms

Microorganisms used in this work are listed in Table 1. *Eremothecium ashbyii* IFO 0952 was obtained from the Institute for Fermentation, Osaka. *Aspergillus oryzae* K (Kawachi) was supplied from Kawachi Co., Kagoshima, *Micrococcus glutamicus* FKU 173 and *Streptomyces griseus* IAM 0084 from laboratory of Industrial Microbiology, Faculty of Agriculture, Kyoto University, and the others from Laboratory of Applied Microbiology, Faculty of Agriculture, Kagoshima University.

Media

The compositions of basal media were as follows. (1) *Bacillus cereus* and *B. subtilis*: 0.5 % meat extract, 1 % peptone and 0.5 % NaCl (pH 7.0). (2) *Chlorella vulgaris, Penicillium chrysogenum* and *Saccharomyces cerevisiae*: a potato extract supplemented with 2 % glucose (pH 7.0). (3) *Leuconostoc mesenteroides*: 5 % glucose, 0.8 % peptone, 0.3 % yeast extract, 0.2 % KH_2PO_4, 0.05 % $MgSO_4 \cdot 7H_2O$, 0.01 % NaCl and $CaCO_3$. (4) *Micrococcus glutamicus*: the medium described by Tanaka *et al.* supplemented with 2.5 μg/ml of biotin[31]. (5) *Aspergillus terreus*: the Lockwood-Reeves medium[25]. (6) *Streptomyces griseus*: the Waksman medium[27]. (7) *A. niger*: the Doelger and Prescott medium[28]. (8) *Eremothecium ashbyii*: 2 % glucose, 1 % peptone, 0.3 % yeast extract, 0.2 % KH_2PO_4, 0.1 % NaCl and 0.01 % $MgSO_4 \cdot 7H_2O$ (pH 5.5)[20]. (9) *A. oryzae* K, *A. kawachii* and *A. awamori*: 5 % glucose, 0.6 % peptone, 0.1 % KH_2PO_4, 0.1 % $MgSO_4 \cdot 7H_2O$, 0.001 % NaCl, 0.001 % $CaCl_2$ and 0.001 % $FeCl_3$ (pH 4.5). Cycasin was added to the basal media at various concentrations as shown in Table 1, followed by autoclaving at 120°C for 10 min.

Cultural conditions

The following microorganisms were submitted to cultivation under the same conditions as described in the literature: *L. mesenteroides*[26], *S. griseus*[27], *M. glutamicus*[31], *A. terreus*[25], *A. niger*[28], and *E. ashbyii*[20]. *B. cereus* and *B. subtilis* were cultivated in culture tubes (1.6 × 20 cm) containing 5 ml of media at 30°C for 3 days by shaking cultivation, and *S. cerevisiae* and *A. awamori* by static cultivation. *C. vulgaris, P. chrysogenum* and *A. kawachii* were cultivated on agar slants at 30°C. *A. oryzae* K was cultivated in Morton tubes (1.6 × 15 cm) containing 5 ml of media at 30°C by static cultivation.

Measurement of growth

Microbial growth was determined by measuring absorbance of culture media at 660 nm or the dry weight of cells.

Determination of fermentation products

Lactic acid was enzymatically determined, using D-lactate dehydrogenase[7]. Itaconic acid, kojic acid and flavins were measured by the methods of Kobayashi and Tabuchi[25], Kawade[12] and Mitsuda *et al.*[20], respectively. Glutamic acid was analyzed with a Yanagimoto SLC-5N amino acid auto-analyzer. Citric acid was determined as total acidity[28].

Determination of cycasin and methylazoxymethanol

Cycasin and methylazoxymethanol were determined by the high-performance liquid chromatography[32].

Determination of protein

Protein was determined by the UV absorption method[21].

Determination of glucose

Glucose was determined with glucomesser reagent.

Measurement of the O_2 uptake by mycelia of A. oryzae K

Mycelium and the culture medium were transferred from a Morton tube into a cell of an Oxygen Uptester (Taiyo Co.). The O_2 uptake was measured at 30°C for a 30-min period, during which it was proportional to time.

Preparation of enzyme solutions from mycelia of A. oryzae K

Mycelia harvested after 13-day incubation in 3 Morton tubes were washed with water, and homogenized with 5 ml of an appropriate buffer in a chilled mortar. The homogenate was centrifuged at 10,000 rpm for 15 min, and 1 ml of the supernatant solution was applied to a small column of Sephadex G-25. The column was developed with the same buffer, and the eluate fractionated. An aliquot of the enzyme fraction was assayed for enzyme activity. On extraction and gel filtration, the following buffers were used : 24 mM triethanolamine-HCl buffer (pH 7.9) containing 6 mM EDTA for triosephosphate isomerase ; 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA for glucose-6-phosphate and phosphogluconate dehydrogenases ; 1/15 M phosphate buffer (pH 7.0) for β-glucosidase and D-glucoside-3-dehydrogenase ; and 0.1 M phosphate buffer (pH 7.0) for glucose oxidase.

Enzyme assays

The activities of the following enzymes in 13-day mycelia were assayed by the methods described in the literature : β-glucosidase[22], p-nitrophenyl-β-D-glucoside being replaced by the o-isomer ; triosephosphate isomerase[9] ; glucose-6-phosphate dehydrogenase[5] ; phosphogluconate dehydrogenase[24] ; D-glucoside-3-dehydrogenase[2] ; and glucose oxidase[3].

Instrumental analyses

Melting points were measured with a Yanagimoto micro melting point apparatus and left uncorrected. UV absorption spectra were recorded on a Hitachi EPS-3T spectrophotometer. IR spectra were taken on a Hitachi EPI-G2 instrument for KBr tablets. PMR spectra were obtained on a JEOL MH-100 spectrometer with dimethylsulfoxide-d_6 as solvent and tetramethylsilane as an internal standard.

Results

Toxic effects of cycasin on various microorganisms

Table 1 shows the effects of cycasin on the growth of various microorganisms and productivities of some fermentation products. Growth of most microorganisms was suppressed by less than 1 % cycasin. *Chlorella* was most sensitive, and bacteria were rather tolerant. In most molds, cycasin also interfered with the formation of spores and pigments. *A. awamori* was not so much affected by 0.2 % cycasin as other molds were. Cycasin inhibited productivities of various substances. The degrees of the inhibition were different to some extent among different strains. *A. oryzae* K was exceptional, because it accumulated an appreciably large amount of a certain substance, positive to the ferric chloride reagent, in the medium on cycasin-treatment. The substance was extracted with diethyl ether from the 14-day culture medium (10 ml), purified by recrystallization from ethanol to give colorless crystals (10 mg), and identified as kojic acid by comparison of the IR spectrum and mp with those of an authentic sample : mp : 150°C ; IR ν_{max}^{KBr} r cm^{-1} : 1664, 1612, 1586, 1232, 1143, 1075, 943, 867 ; NMR $\delta_{Me_4Si}^{DMSO}$: 4.30 (2H, doublet, -CH_2-OH), 5.62 (1H, triplet, -CH_2-OH), 6.34 (1H, singlet, =CH-), 7.98 (1H, singlet, =CH-), 8.96 (1H, singlet, -OH).

Table 1.   Effect of cycasin on various microorganisms

Microorganism	Cycasin in media (%)	Growth[*1] (%)	Fermentation product	
			Compound	Amount (%)[*1]
Bacillus cereus IFO 3001	0.1	No effect[*2]		
	1.0	Autolysis		
Bacillus subtilis IFO 3007	0.1	No effect[*2]		
	1.0	60[*2]		
Leuconostoc mesenteroides B07	0.1	—	Lactic acid	10
Streptomyces griseus IAM 0084	1.0	60[*3]		
Micrococcus glutamicus FKU 173	0.2	60[*2]	Glutamic acid	20
Saccharomyces cerevisiae	0.1	No effect[*2]		
IAM 4702	1.0	70[*2]		
Penicillium chrysogenum IFO 4626	1.0	Inhibition of growth[*4] and spore-formation		
Aspergillus niger	0.1	Inhibition of growth (60[*3]) and spore-formation	Citric acid	60
	1.0	No growth		
Aspergillus terreus 4100	0.05	33[*3]	Itaconic acid	40
Aspergillus kawachii IFO 4308	1.0	Inhibition of growth[*4] and spore-formation		
Aspergillus oryzae K (Kawachi)	0.2	Inhibition of growth (40[*3]) and spore-formation	Kojic acid	600
Aspergillus awamori IFO 4314	0.2	No effect on growth[*3] and spore-formation		
Chlorella vulgaris IAM C-30	0.2	No growth[*4]		

[*1] The figures represent the ratio of cycasin group to the control (%).

[*2] Turbidity at 660 nm.   [*3] Dry weight of cells.   [*4] Observation on agar slants.

Effects of cycasin on A. oryzae K

Growth, the pH of the culture medium and the production of kojic acid were compared between mycelia of *A. oryzae* K cultivated in the basal medium with and without 0.2 % cycasin (Fig. 1). Growth of cycasin-treated mycelia was similar to that of the control until day 6, but thereafter it did not increase. The dry weight of cycasin-treated mycelia on day 20 was 40 % of that of the control. Spore-formation was also inhibited by cycasin. Accumulation of kojic acid by cycasin-treated mycelia began to be significant after day 6. The maximum amount of kojic acid in the culture medium, attained on day 20, was about 6 times that of the control. A lowering of the pH of the culture medium was not so appreciable on cycasin-treatment.

Glucose consumption of cycasin-treated mycelia was much more significant than that of the control after day 8 (Fig. 2). Cycasin was decomposed by mycelial β-glucosidase to
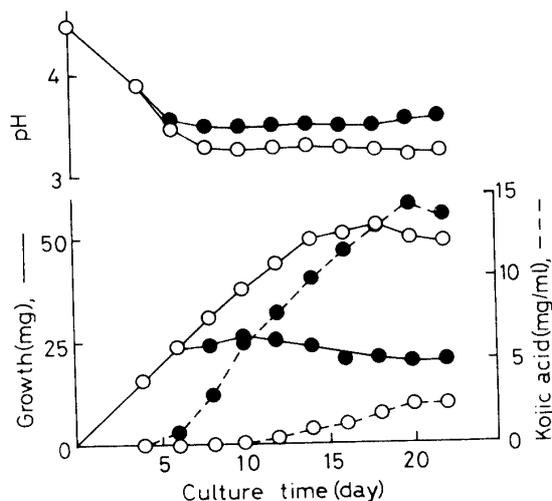
Fig. 1. Effect of cycasin on growth of *A. oryzae* K, production of kojic acid and pH of culture medium.

A. oryzae K was cultivated in Morton tubes containing 5 ml of the basal medium with and without 0.2 % cycasin at 30°C by static cultivation. Mycelia were harvested on the days 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22, and the dry weight was measured (—○—, control ; —●—, cycasin). An appropriate volume of the culture medium was analyzed for kojic acid (⋯○⋯, control ; ⋯●⋯, cycasin) and the pH of the culture medium (—○—, control ; —●—, cycasin). Results are expressed as the means of 5 determinations.
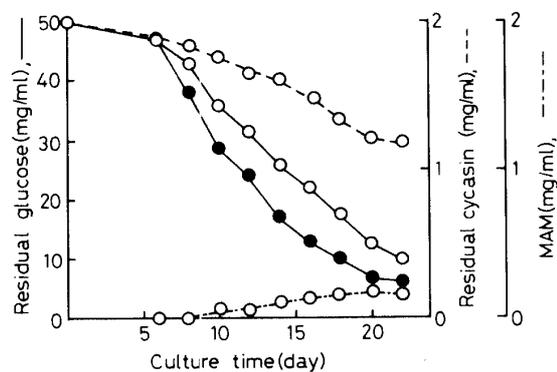
Fig. 2. Effect of cycasin on consumption of glucose and degradation of cycasin.

A. oryzae K was cultivated as described in Fig. 1. An appropriate volume of the culture medium was analyzed for glucose (—○—, control ; —●—, cycasin), cycasin (⋯○⋯) and methylazoxymethanol (—○—). Results are expressed as the means of 5 determinations.

liberate methylazoxymethanol during the cultivation period.

The effects of cycasin on growth of mycelia and production of kojic acid were dependent on cycasin concentration (Fig. 3). The yield of kojic acid to the consumed sugar was maximal in the range of 0.2 to 0.5 % cycasin.
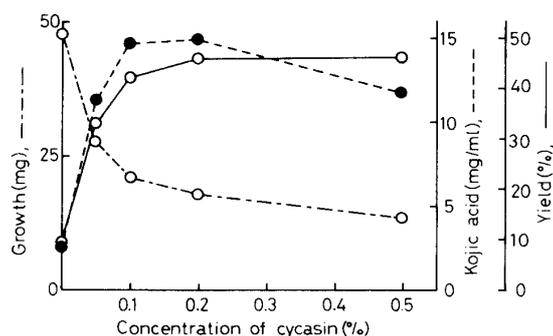
The O_2 uptake of cycasin-treated mycelia was more enhanced between day 4 and 10 than that of the control (Fig. 4). When respiration was expressed as μl O_2 uptake/min·mg dry weight of mycelium, respiration of cycasin-treated mycelia was about 1.3-1.6 times that of the control during the cultivation period after day 6.

After day 20, kojic acid decreased with concomitant formation of an unidentified compound positive to the ferric chloride reagent. The compound was isolated as follows. The 27-day culture medium (100 ml) was adjusted to pH 3 with dil. HCl and applied to a small column of charcoal. ·The compound was eluted with a linear gradient elution of 0-60 % ethanol. The fraction concerned was evaporated to dryness, and the residue was repeatedly crystallized from water to give colorless needles (10 mg). The compound was identified as 2, 2′-dihydroxymethyl-6, 6′-dikojylmethane : its mp and IR spectrum were identical with those of an authentic sample prepared by the method of Ichimoto et al.[10] : mp : 250°C ; UV λ_{max}^{MeOH} nm : 286 ; IR ν_{max}^{KBr} cm^{-1} : 3100, 2900, 2850, 1660, 1630, 1620, 1580, 1460, 1310, 1230, 1090, 1050, 1000, 880, 860, 760 : NMR $\delta_{Me_4Si}^{DMSO}$: 4.04 (2H, singlet, -CH$_2$-), 4.28 (4H,

Fig. 3. Effect of cycasin concentration on growth of *A. oryzae* K, production of kojic acid and yield of kojic acid.

A. oryzae K was cultivated as described in Fig. 1 with the exception of the various concentrations of cycasin. Mycelia were harvested on day 20 and measured (— - -○- - -—). An appropriate volume of the culture medium was analyzed for kojic acid (--- ●---). The yield was estimated as molar ratio of kojic acid to the consumed glucose (— ○ —). Results are expressed as the means of 3 determinations.
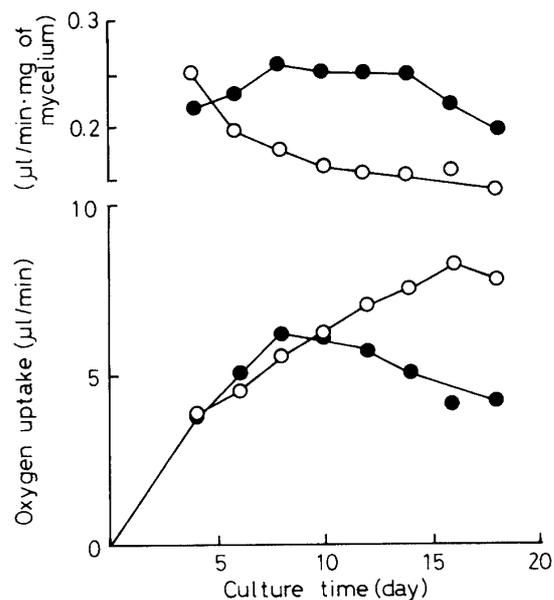

Fig. 4. Effect of cycasin on O₂ uptake of mycelium of *A. oryzae* K.

A. oryzae K was cultivated as described in Fig. 1. Mycelium and culture medium were placed in a cell of an Oxygent Uptester. The O₂ uptake was measured at 30°C (—○—, control ; —●—, cycasin), and expressed as μl O₂/min·mycelium and μl O₂/min·mg dry weight of mycelium. Results are expressed as the means of 3 determinations.

Table 2. Effect of cycasin on activities of several enzymes in mycelium of *A. oryzae* K[1]

Enzyme	Specific activity (unit/mg protein)[2]	
	Control	Cycasin-treated
β-Glucosidase	0.14	0.12
Triosephosphate isomerase	3.05	0.17
Glucose-6-phosphate dehydrogenase	0.06	0.08
Phosphogluconate dehydrogenase	0.019	0.012
D-Glucoside-3-dehydrogenase[3]	ND[4]	ND
Glucose oxidase	ND	ND

[1] The figures are the means of 2 determinations.

[2] One unit of β-glucosidase activity was defined as the amount of enzyme catalyzing the liberation of 1 μmole of *o*-nitrophenol per min from its glucoside. For the enzymes other than β-glucosidase, unit was defined as described in the literature.

[3] Glucose, glucose-6-phosphate and glucose-1-phosphate were used as substrates.

[4] Enzyme activity was undetectable under the conditions used.

doublet, $2 \times$-CH$_2$-OH), 5.57 (2H, triplet, $2 \times$-CH$_2$-OH), 6.30 (2H, singlet, $2 \times$=CH-), 8.93 (2H, singlet, $2 \times$-OH).

Effects of cycasin on activities of enzymes in mycelia

The activities of several enzymes in 13-day mycelia of *A. oryzae* K were analyzed for the mycelial extracts (Table 2). Triosephosphate isomerase activity was markedly decreased on cycasin-treatment, whereas other enzymes were not so much affected. As shown in rice plants[30], cycasin did not seem to interfere with the synthesis of enzymes in a random fashion.

Discussion

The carcinogenic activity of cycasin was suggested to be due to its aglycone, methylazoxymethanol, acting as an alkylating agent for nucleic acids and/or proteins[19]. Cycasin interfered with seed germination, seedling growth and formation of α-amylase in rice endosperms[30]. In this work, cycasin was presumed to show toxic effects on microorganisms. Cycasin inhibited the growth of various microorganisms; productivities of some fermentation products; and spore-formation of molds (Table 1). Toxicity was observed at an concentration of less than 1 %. *A. oryzae* K was different from other microorganisms in the increased accumulation of a fermentation product, kojic acid, on cycasin-treatment (Fig. 1). The effects of cycasin on this mold were, therefore, examined in connection with the accumulation of kojic acid in the culture medium. Growth of mycelia was completely suppressed by 0.2 % cycasin after day 6 (Fig. 1), whereas consumption of glucose (Fig. 2), production of kojic acid (Fig. 1) and the O$_2$ uptake (Fig. 4) were greatly increased as compared with those of the control. These results can be interpreted as indicating that a substantial amount of glucose was used for the formation of kojic acid, but not for growth of cells, on cycasin-treatment. In the later period of cultivation, a part of kojic acid was converted into 2,2'-dihydroxymethyl-6,6'-dikojylmethane. Ichimoto *et al.*[10] reported that this compound was formed in an organic reaction of kojic acid with formaldehyde. We infer that the accumulation of the compound in the culture medium is due to the reaction described above, because formaldehyde is released from methylazoxymethanol as one of its decomposition products.

Two pathways are proposed for the biosynthesis of kojic acid, but not yet established. The main pathway is the direct oxidation of glucose without cleavage of the carbon chain[1,13], and the other is the condensation of triose (s) such as dihydroxyacetone (phosphate)[1,4,6,11]. Glucose-6-phosphate dehydrogenase, D-glucoside-3-dehydrogenase or glucose oxidase is presumed to be situated in the former pathway[1]. Cycasin was assumed to raise the activities of these three enzymes, but not so great a difference was detected between the control and cycasin-treated mycelia. On the other hand, triosephosphate isomerase activity was markedly decreased on cycasin-treatment. In a preliminary experiment using paper chromatography, dihydroxyacetone and its phosphate were not shown to accumulate in the culture medium or mycelia. These results suggest that the formation of kojic acid by condensation of dihydroxyacetone (phosphate) was responsible for the increased accumulation of kojic acid in the culture medium on cycasin-treatment. However, we can not rule out a possibility that the decrease of triosephosphate isomerase activity disturbed glycolysis and that glucose was mainly converted into kojic acid by the direct oxidation. Further studies are necessary to elucidate the mechanism of stimulation of kojic acid formation by cycasin.

Summary

Cycasin, a toxic glycoside of cycad plants, inhibited the growth of various microorganisms; productivities of some fermentation products; and spore-formation of molds. Toxicity was observed at an concentration of less than 1 %. *Aspergillus oryzae* K (Kawachi) differed from other microorganisms in the increased accumulation of a fermentation product, kojic acid, in the culture medium on cycasin-treatment.

The effects of cycasin on *A. oryzae* K were examined in connection with the accumulation of kojic acid. Although growth of cells was suppressed by cycasin, consumption of glucose, production of kojic acid and the O_2 uptake were greatly increased on cycasin-treatment, indicating that a substantial amount of glucose was used for the formation of kojic acid, but not for growth of cells, on cycasin-treatment. There was no significant difference between the control and cycasin-treated mycelia in the activities of enzymes in mycelia except for triosephosphate isomerase.

Acknowledgments

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