Formation Mechanism for Potent Antioxidative 6-Dihydroxyisoflavones in Soybeans Fermented with Aspergillus saitoi

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Received October 29, 1998; Accepted January 27, 1999

The formation mechanism for the potent antioxidative 6-dihydroxyisoflavones, 8-hydroxydaidzein (8-OHD) and 8-hydroxyisoflavanogen (8-OHG), was studied by incubating whole soybeans in a solid culture and a soybean extract in a liquid culture with Aspergillus saitoi.

Analyses of changes in the isoflavone analogue content, β-glucosidase activity, and isoflavone hydroxylation ability indicated that 8-OHD and 8-OHG were formed from daidzein and genistein, respectively, by microbial hydroxylation, being respectively liberated from daidzin and genistin by β-glucosidase from A. saitoi during incubation. No selective hydroxylation reaction at the 8-position of daidzein and genistein were apparent during the vegetative stage, but were induced at the stage of sporulation.

Key words: antioxidant; 8-hydroxydaidzein; 8-hydroxyisoflavanogen (8-OHG); Aspergillus saitoi; sporulation

Polyphenolic compounds are widely distributed in plant foods (legumes, cereals, vegetables, fruits, etc.) and in their products (soybean products, spices, tea, wine, etc.).1-8 Recent studies have demonstrated that the intake of dietary antioxidants, including these polyphenols, had beneficial effects on preventing cancer and other diseases.9-13 The consumption of polyphenol-rich foods has been associated with a lowered risk of mortality from coronary heart disease in elderly men.14 Soybeans, which are recognized as being a good source of several nutrients, contain many kinds of polyphenols.1,5 The main polyphenols are isoflavone analogues such as daidzin, genistin, daidzein, and genistein. As another polyphenols, α-, γ-, and δ-tocopherols, saponins, chlorogenic acid isomers, caffic acid, and ferulic acid are also contained. These polyphenols have the potential abilities to scavenge free radicals related to oxygen and to chelate metal ions.

Therefore, they also have the possibility of bringing many antioxidative and biological activities to the human body, in addition to retarding oxidative racidity in processing and preservation.

Soybeans are usually eaten as processed foods (tofu, natto, tempeh, miso, shoyu, etc.). It is interesting to note that fermented soybean foods produced by using microorganisms do not lose their antioxidative properties, but in fact show increased antioxidative activity.16-18 In our laboratory, studies on antioxidants in fermented soybean products have been carried out. 3-Hydroxyxanthranilic acid was isolated and identified from tempeh as the principal antioxidant which was formed during fermentation and that increased the antioxidative activity of tempeh.19 We have recently isolated two potent antioxidative isoflavones from soybeans fermented with A. saitoi.20 These isoflavones, 8-hydroxydaidzein (8-OHD) and 8-hydroxyisoflavanogen (8-OHG), have an o-dihydroxy structure at the 7- and 8-position, were produced during fermentation/incipubation with A. saitoi. They showed more stronger antioxidative activities than those of daidzein and genistein, respectively.

In this study, to make clear the formation process and mechanism of 8-OHD and 8-OHG during fermentation/incipubation with A. saitoi, we examined the changes in isoflavone analogues and enzymatic activities of β-glucosidase (EC 3.2.1.21) in addition to the isoflavone hydroxylation enzyme (hydroxylase) at the 8-position by using samples prepared from both solid and liquid cultures.

Materials and Methods

Chemicals. Soybean oil, p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenol, and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries, Osaka, Japan. The other chemicals used in the previously reported study20 were also utilized in this study.

Detection and evaluation of the antioxidative substances. A preliminary detection and evaluation of the antioxidative substances was made by a slightly modified TLC-fluorescent method.21 A fluorescent TLC plate (Merck 1.05715) spotted with a sample was developed with toluene:ethyl formate:formic acid (10:8:2, v/v). The plate was sprayed with a tocopherol-stripped soybean oil solution of ca. 5% concentration in n-hexane, before being exposed to short-wave UV irradiation (254 nm). The entire background gradually turned dark,
while the spots of the antioxidative substances remained unchanged, resulting in the appearance of bright fluorescent spots on a dark background. A preliminary evaluation of the antioxidative activity of each substance was performed by recording the time for the disappearance of each fluorescent spot.

The total antioxidative activity of each sample was also determined in a liposome system as previously reported.\textsuperscript{20}

\textbf{Assays of \( \beta \)-glucosidase and hydroxylase activities.} The \( \beta \)-glucosidase activity was determined by using a synthetic substrate, \textit{pNPG}, by the method of Matsuura \textit{et al.}\textsuperscript{23} \textit{pNPG} (2.0 ml, 1 mM) in a 0.1 M phosphate-citrate buffer (pH 5.0) was pre-incubated at 30°C for 5 min. After the addition of 0.5 ml of an enzyme solution, the reaction mixture was incubated at 30°C for 60 min. The reaction was stopped by adding 2.5 ml of 0.5 M sodium carbonate. The resulting \( \textit{p} \)-nitrophenol was immediately monitored at 420 nm with a Shimadzu UV-160A spectrophotometer and its amount was calculated from the standard curve. One unit of \( \beta \)-glucosidase is defined as the amount of enzyme which liberated 1 \textmu mol of \textit{p}-nitrophenol per min.

The isoflavone hydroxylase activity was measured by using genistein as a substrate by the HPLC method of Cheynier \textit{et al.}\textsuperscript{20} Genistein (20 \textmu l, 5 mM) in DMSO was mixed with 0.48 ml of a 0.1 M phosphate buffer (pH 5.0). After adding 0.5 ml of an enzyme solution, the reaction mixture was incubated at 30°C. At intervals, the reaction mixture (200 \textmu l) was taken and mixed with 200 \textmu l of ethanol, before being stored at \(-30 ^\circ C\) until needed for analysis. The amounts of residual genistein and newly formed hydroxylated genistein (8-OHG) in the reaction mixture were determined by HPLC. HPLC was performed in a Develosil ODS-UG-5 column (Nomura, 4.6 i.d. \times 250 mm), using methanol-water (45:55 v/v) containing 0.1% (v/v) TFA as the solvent at a flow rate of 0.7 ml/min and monitoring by UV (262 nm, Hitachi, 655A-2). Each peak was identified by using a threedimensional HPLC system equipped with a photodiode array detector.\textsuperscript{20} One unit of hydroxylase is defined as the amount of enzyme which produced 1 nmol of 8-OHG per hour under these assay conditions.

\textbf{Detection of the antioxidative substances in soybeans fermented with \textit{A. saitoi} for different periods.} Fermented soybeans incubated with \textit{A. saitoi} for 0, 1, 2, 3, 4 and 5 d\textsuperscript{20} were used in this study. Samples of lyophilized soybean powder (500 mg) after different fermentation periods were extracted with methanol (5.0 ml). Each extracted solution (10 \textmu l) was employed for detection of the antioxidative substances by the TLC-fluorescent method already described.

\textbf{\( \beta \)-glucosidase assays of the soybean samples fermented for different periods.} The same samples of lyophilized soybean powder (10 mg) were mixed with 5.0 ml of a 0.1 M phosphate-citrate buffer (pH 5.0), and each mixture was thoroughly suspended by being treated with an ultrasonic vibrator (Branson model B-12). Aliquots (0.5 ml) of the suspension and/or a diluted suspension were used as an enzyme solution for the assay of \( \beta \)-glucosidase activity already described. The soybean powder was removed by centrifugation (2,200 \times g, 10 min) just before being monitored at 420 nm.

In addition, each sample of lyophilized powder (1.0 g) was extracted with 12 ml of a 0.1 M phosphate-citrate buffer (pH 5.0) at 4°C for 16 h while continuously shaking. The enzyme solution was collected by centrifugation (2,200 \times g, 10 min). The \( \beta \)-glucosidase activity was determined by using the supernatant.

\textbf{Incubation of genistein with the soybean samples fermented for different periods.} Each lyophilized sample of soybean powder (100 mg) from different fermentation periods was thoroughly suspended in 1.0 ml of a 0.01 M phosphate buffer (pH 5.0). After adding 40 \textmu l of 5 mM genistein in DMSO and 0.96 ml of the 0.1 M phosphate buffer (pH 5.0), the mixture was incubated at 30°C for 8 h while continuously shaking. The reaction was stopped by adding 2.0 ml of ethanol. The supernatant recovered by centrifugation was analyzed to determine the amount of 8-OHG by HPLC under the same conditions as those already described. As a blank experiment, each sample of lyophilized soybean powder (100 mg) was suspended in 1.0 ml of ethanol and then 40 \textmu l of DMSO and 0.96 ml of the 0.1 M phosphate buffer were added. After the addition of ethanol, the amount of 8-OHG was determined by HPLC.

\textbf{Cultivation of \textit{A. saitoi} with the soybean extract.} Whole soybeans\textsuperscript{20} (400 g) were soaked overnight in 1.6 l of water at room temperature. The mixture was heated at 105°C for 60 min and then filtered through gauze. The resulting filtrate was centrifuged to remove the small particles. Aliquots (19.5 ml) of the soybean extract in 100-ml Erlenmeyer flasks were autoclaved at 121°C for 15 min. Three flasks were stored as samples from day 0. The other flasks, which had been inoculated with 0.5 ml of the suspension of \textit{A. saitoi} conidia (1.60 \times 10^6 spores per ml), were incubated at 30°C by using a rotary shaker (140 rpm/min, Thomas TAL-RS12). The first 3 flasks were withdrawn from the incubator after 0.6 d, and the second 3 flasks after 1.0 d. The other flasks incubated with shaking were allowed to grow further in the stationary state at 30°C with no shaking, the third 3 flasks being withdrawn after 1.6 d, the fourth 3 flasks after 2.6 d, and the other 3 flasks after 3.6 d. Each flask collected after the different incubation periods was immediately treated. Mycelia from the flask were harvested by being passed through a 3G-2 glass filter, and the resulting culture filtrate was centrifuged at 4°C to remove small particles and/or spores. Each supernatant subdivided in small tubes was immediately kept at \(-80 ^\circ C\). The mycelia were washed thoroughly with a cold physiological saline solution, lyophilized, and the mycelial yield was determined. The mycelia after 3.6 d could not harvested because it was impossible to wash due to its autolysis. Each lyophilized material was ground and stored at \(-80 ^\circ C\) until needed for analysis.
Analysis of each culture filtrate incubated for different periods. The pH value of each culture filtrate was measured with a Horiba M-7iPrH meter. A 3.0-ml aliquot of the filtrate was lyophilized, and the resulting dried material was extracted with 2.0 ml of 70% methanol. Each extracted solution (1.0 ml) was diluted with the same solvent to 10 ml, and then each diluted sample (100 μl) was used for the analysis of antioxidative activity in the liposome system already described. A control experiment without a sample was conducted by using 100 μl of 70% methanol at the same time.

The antioxidative substances in each culture filtrate were examined by the TLC-fluorescent method already described. Each filtrate (6.0 ml) after a different incubation period was lyophilized, and the resulting materials were each extracted with methanol (0.4 ml). Each extracted solution (10 μl) was spotted on a TLC plate.

Quantitative analyses of 8-OHD, 8-OHG, and the other isoflavone analogues in each culture filtrate incubated for different periods were performed by HPLC. A 1.0-ml aliquot of a filtrate was mixed with 1.0 ml of ethanol, and the mixture was centrifuged at 2,200 × g for 10 min. The supernatant (10 μl) was subjected to HPLC performed on a Develosil ODS-UG-5 column (Nomura, 4.6 i.d. × 250 mm), using methanol-water (45:55 v/v) containing 0.1% (v/v) TFA as the solvent at a flow rate of 0.7 ml/min and monitoring by UV (262 nm, Hitachi, 655A-2). Each peak was identified by using the three-dimensional HPLC system.

The culture filtrates were also used for the assays of β-glucosidase and hydroxylase activities already described. The total activity (units) of each of these enzymes in a culture flask was calculated as a 20-ml volume of the culture filtrate.

β-glucosidase and hydroxylase assays of the mycelia. The membrane-bound β-glucosidase and hydroxylase activities were determined by using a lyophilized mycelial powder. Each material (5.0 mg) harvested after different incubation periods was thoroughly suspended in 0.5 ml of a 0.1 M phosphate-citrate buffer (pH 5.0), and the neat suspension or a diluted one was used as the enzyme solution for the assay of β-glucosidase activity already described. The mycelial powder was removed by centrifugation (2,200 × g, 10 min) just before being monitored at 420 nm. The total activity of β-glucosidase in a culture flask was calculated on the basis of the total mycelial yield harvested from each culture flask.

Another 2.5 mg of each mycelial powder was also suspended in 0.5 ml of a 0.1 M phosphate buffer (pH 5.0), and the resulting suspension was utilized as an enzyme solution for determining the hydroxylase activity in mycelia incubated for different periods. In addition, the same suspension was boiled at 100°C for 10 min, and the heated material was also assayed. The total activity of hydroxylase in a culture flask was also calculated on the basis of the total mycelial yield harvested from each culture flask.

Statistics. A statistical analysis was performed by using Student's t-test.

Results and Discussion
Appearance of 8-OHD and 8-OHG in soybeans fermented for different periods
Steam soybeans were fermented with A. saitoi for 1, 2, 3, 4 and 5 d. Vegetative mycelia grew, and the soybeans were covered with white mycelia after 1 d. After 2 d of fermentation, the mycelia had started their sporulation. This sporulation progressed with increasing fermentation period. The antioxidative substances in methanol extracts prepared from the soybeans fermented for different periods were investigated by the TLC-fluorescent method, the results being shown in Fig. 1. Three main kinds of antioxidative spot were detected at Rf values of 0.45, 0.60 and 0.67. The spots at Rf 0.45 and 0.60 were identified as 8-OHD and 8-OHG, respectively, by comparing their Rf values with those of authentic samples. These two potent antioxidants appeared after 2 d of fermentation, which was the starting stage of sporulation. On the other hand, the compound corresponding to an Rf value of 0.67, which is an unidentified antioxidant, was produced after 1 d.

In the previous paper,20 it was reported that daidzin and genistin in steamed soybeans decreased with fermentation period, whereas daidzein and genistein, aglycones of daidzin and genistin, respectively, increased during fermentation. Daidzin and genistin also have a β-glucosidic linkage at the 7-position in the A ring of the corresponding isoflavone aglycones, namely daidzein and genistein, respectively. Hydrolytic enzymes (β-glucosidase) of soybean isoflavone glucosides have been reported from Rhizopus oryzae,9 Rhizopus oligosporus,20 Lac-

![Fig. 1. Thin-layer Chromatogram of Antioxidative Substances in Methanol Extracts from Soybeans Fermented with A. saitoi for Different Periods.](Image)
tobacillus casei,20 and raw soybeans.23 However, no studies on the β-glucosidase of Aspergillus saitoi have been reported. It is thus necessary to confirm that daidzin and genistin were hydrolyzed into daidzein and genistein, respectively, by β-glucosidase produced from the A. saitoi fermentation. The β-glucosidase activities of soybeans fermented for different periods were investigated. In the first experiment, the enzyme activity was determined by using a suspension of each fermented soybean powder. Furthermore, it was also assayed for extracted solutions of the same powders. As shown in Fig. 2, both β-glucosidase activities gradually increased with fermentation, being especially enhanced after 2 d, which was the stage of sporulation. These results prove the conversion of daidzin and genistin into daidzein and genistein, respectively, during the soybean fermentation described in Fig. 7 of the previous paper.20

8-OHD and 8-OHG were selectively hydroxylated at the 8-position of each corresponding isoflavone, daidzein and genistein. To confirm these reactions, the converting potential of genistin into 8-OHG was investigated by using samples of the soybean powder fermented for different periods. Blank experiments were also done, since some samples of soybean powder after different fermentation periods that were used as enzyme materials in this test had contained the original 8-OHG. These results are shown in Table 1. In this table, the newly produced amounts (μg/4.0 ml of the reaction mixture) of 8-OHG during the incubation for 8 h are given in column C. This table elucidates that genistin was converted into 8-OHG by the soybeans fermented for 2, 3, 4 and 5 d, which are in the sporulation stage, whereas this conversion didn’t arise at 0 and 1 d.

These results suggest that 8-OHD and 8-OHG were formed by daidzin and genistin in steamed soybeans being gradually converted into the corresponding aglycones, daidzein and genistein, respectively, by β-glucosidase from the A. saitoi fermentation. These resulting free isoflavones were then hydroxylated enzymatically to form the potent antioxidants, 8-OHD and 8-OHG.

**Growth of A. saitoi in a liquid culture of a soybean extract**

In the former experiments, 8-OHD and 8-OHG were produced in the fermented soybeans at the stage of sporulation, whereas they were not detected during vegetative growth. However, vegetative mycelia continued to grow after even 2 d of fermentation in a solid culture when steamed whole soybeans were used.

This experiment was designed to grow only vegetative mycelia to suppress the sporulation of A. saitoi in a liquid culture when using the soybean extract. The submerged culture was initially grown by using a rotary shaker for 1.0 d, and subsequently surface-cultured to promote sporulation predominantly in a stationary condition for a further 2.6 d. Only vegetative hyphae were observed in pellet form for periods up to 1.0 d. The mycelia and culture filtrates at the stages of 0.6 and 1.0 d were collected.

Further incubation for 0.6 d (total of up to 1.6 d) of vegetative hypha in the stationary phase promoted slight sporulation. The incubation was continued for 1.6 and 2.6 d (total of up to 2.6 and 3.6 d). The sporulation which was incubated for 3.6 d in total was almost complete.

The yields of resulting mycelia and pH values of the culture filtrates are shown in Table 2. The amount of mycelia increased markedly up to 1.0 d and continued to increase gradually from 1.0 d to 2.6 d. The pH of the filtrate gradually dropped with incubation time during 2.6 d. The initial pH of a soybean extract was 6.0, and the pH of the filtrate after 2.6 d was 3.1.

**Table 1. Conversion Ability of Genistin into 8-OHG by Soybean Powder Samples Fermented for Different Periods**

<table>
<thead>
<tr>
<th>Fermentation period (days)</th>
<th>8-OHG (μg/4.0 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>13.2</td>
</tr>
<tr>
<td>3</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>16.6</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Each value is the mean of duplicates.

A: Amount of the original 8-OHG (μg/100 mg of fermented soybean powder) which had been produced during the fermentation of soybeans with A. saitoi for different periods.

B: Amount of total 8-OHG which was produced from the reaction mixture of the fermented soybean powder (100 mg) at different stages with added genistein (54 μg) during the incubation at 30°C for 6 h.

C: Amount of 8-OHG which was newly produced from the reaction mixture by the addition of genistein.

**Fig. 2. Changes of β-Glucosidase Activities in Soybeans during Fermentation.**

β-Glucosidase activity was assayed in a 0.8 mM pNPG and 0.1 M phosphate-citrate buffer (pH 5.0), using a soybean suspension (●) and extracted solution (▲). Details are given in the Materials and Methods section. Symbols in this figure are the means of duplicates.
Table 2. Changes of Mycelial Yield, pH of the Culture Filtrate, and Growth State in a Liquid Culture of a Soybean Extract

<table>
<thead>
<tr>
<th>Incubation period (days)</th>
<th>Mycelial yield (mg dry wt.)</th>
<th>pH (culture filtrate)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6.00±0.01</td>
<td>None</td>
</tr>
<tr>
<td>0.6</td>
<td>28.7±5.79</td>
<td>5.70±0.05</td>
<td>Vegetative mycelia</td>
</tr>
<tr>
<td>1.0</td>
<td>166±13.3*</td>
<td>4.70±0.01</td>
<td>Vegetative mycelia</td>
</tr>
<tr>
<td>1.6</td>
<td>231±4.48</td>
<td>3.90±0.01</td>
<td>Sporulation</td>
</tr>
<tr>
<td>2.6</td>
<td>285±11.5</td>
<td>3.10±0.09</td>
<td>Sporulation</td>
</tr>
<tr>
<td>3.6</td>
<td>*</td>
<td>4.20±0.13</td>
<td>Sporulation</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (n=3).
* P<0.01, compared with 0.6 day.
1 Each mycelial yield was determined from the total amount of lyophilized mycelia recovered from a culture flask.
* Mycelia were not collected because of autolysis.

Antioxidative activities of the culture filtrate

The antioxidative activities of the culture filtrates incubated for different periods were determined in a liposome system. The lipid oxidation level of each sample was set at 100% for the soybean extract at the start of incubation. As shown in Fig. 3, the antioxidative activity gradually increased during 3.6 d of incubation with A. saitoi. In particular, the activity from 1.6 d, the starting point of sporulation, was significantly (P<0.01) stronger than that of after 0.6 and 1.0 d. This relationship be-

tween the antioxidative activity and the stage of sporulation was compatible with that when A. saitoi was directly incubated with steamed whole soybeans.20)

In addition, the antioxidative substances in each culture filtrate was examined by the TLC-fluorescent method. Potent antioxidative spots, 8-OHD (Rf=0.45) and 8-OHG (Rf=0.60), were characteristically detected in the samples of 1.6, 2.6, and 3.6 d. This phenomenon is in agreement with that when A. saitoi was directly incubated with steamed whole soybeans as shown in Fig. 1. The other antioxidative spot corresponding to an Rf value of 0.67 was characteristically detected in the culture filtrate after 1.0 d. This antioxidant could have been responsible for the antioxidative enhancement after 1.0 d in Fig. 3.

Changes of 8-OHD, 8-OHG, daidzein, genistein, and their glucosides contents in the culture filtrate

In further studies, the amounts of 8-OHD, 8-OHG, and other isoflavone analogues in the culture filtrates incubated for different periods were investigated by HPLC. In addition, these isoflavones were identified by the three-dimensional HPLC method by comparing their elution times and UV spectra for the peaks with those of authentic samples. As shown in Fig. 4, daidzin and genistin, which predominantly existed in the soybean extract at day 0, decreased with increasing incubation time, and these components reached approximately the zero level after 2.6 d. The rate of decrease of these isoflavone glucosides significantly increased (P<0.01) at 1.6

Fig. 3. Antioxidative Activities of Culture Filtrates Incubated for Different Periods. A 70% methanol extract (100 μl) from each culture filtrate was employed for the antioxidative assay in a liposome system as described in the Materials and Methods section. The lipid oxidation level of each sample was set at 100% for the soybean extract at day 0 of incubation. Each value in this figure is the mean ± SD (n=3). (a) P<0.01, compared with 0.6 and 1 d. (b) P<0.01, compared with 0.6 and 1 d. (c) P<0.01, compared with 0.6 and 1 d.

Fig. 4. Changes of Isoflavone Glucosides, Aglycones, and 8-OHD and 8-OHG Contents in the Culture Filtrates during Incubation. Each isoflavone content in the culture filtrates incubated for different periods was determined by HPLC as described in the Materials and Methods section. Each value in this figure is the mean ± SD (n=3). The symbols used in this figure are as follows: ○ genistin; ● daidzin; ● daidzein; ◻ 8-OHD; and ◼ 8-OHG. (a) P<0.01, compared with 1 d. (b) P<0.01, compared with 1 d.
d, which was the starting stage of sporulation.

On the other hand, daidzein and genistin, which are aglycones of daidzin and genistin, respectively, gradually increased during the incubation with *A. saitoi*, and each reached a maximum content after 1.6 d. In the culture filtrate incubated for 2.6 d, daidzin and genistin may have been completely converted into daidzein and genistein. However, the formed amounts of both daidzein and genistein at 2.6 d were stoichiometrically too low.

In contrast, 8-OHD and 8-OHG, which are each selectively hydroxylated at the 8-position in the daidzein and genistein structures, respectively, appeared from the stage of 1.6 d at the start of sporulation. These isoflavones hydroxylated at the 8-position are known to show very strong antioxidative activity, and moreover, they markedly increased in accordance with the progress of sporulation. These results are shown by the change in antioxidative activity during incubation in Fig. 3. This phenomenon has also been observed when *A. saitoi* was incubated on steamed whole soybeans as a solid medium. The amount of 8-OHD after 3.6 d was ca. 0.3 mg per 20 ml of culture filtrate. In addition, 8-OHG reached its maximum content (ca. 0.5 mg/20 ml of culture filtrate) after 2.6 d. On the other hand, neither 8-OHD and 8-OHG were detectable from either culture filtrate incubated for 0.6 and 1.0 d, which were at the growing stage of only vegetative mycelia.

**β-Glucosidase activities of the mycelia and the culture filtrates incubated for different periods**

The results in Fig. 4 suggest that daidzin and genistin in the soybean extract were converted into daidzein and genistein, respectively, by β-glucosidase produced from *A. saitoi*.

In general, *Aspergillus* species are known to produce both intracellular and extracellular β-glucosidases. Therefore, the enzyme activities were determined by using both the mycelia and culture filtrates incubated for different periods. These results are shown in Fig. 5. The total activities of β-glucosidase in culture filtrates incubated for 0.6, 1.0, 1.6, 2.6, and 3.6 d were 0.70, 1.60, 2.60, 32.7, and 748 units/20 ml, respectively. It follows that the extracellular β-glucosidase activity gradually increased during 1.6 d of incubation with *A. saitoi*, and was greatly enhanced after 2.6 d during the progressive stages of sporulation. In addition, the intracellular β-glucosidase activity of the mycelia also gradually increased during 1.6 d of incubation; the activity after 0.6, 1.0 and 1.6 d was 0.18, 2.79 and 8.44 units/total mycelia, respectively, the enhancement of activity after 2.6 d being greater than that of the extracellular culture filtrate. These results agree well with the decreases of daidzin and genistin in Fig. 4. In this study, β-glucosidase activity was determined by using pNPG. However, it has been confirmed that the mycelia could efficiently hydrolyze daidzin and genistin to form daidzein and genistein, respectively (Esaki, H. and Kawakishi, S., unpublished results).

![Fig. 5. Changes of β-Glucosidase Activities in the Culture Filtrates and Mycelia during Incubation.](image)

**Table 3. Changes of Isoflavone Hydroxylase Activities in the Culture Filtrates and Mycelia Incubated for Different Periods**

<table>
<thead>
<tr>
<th>Incubation period (days)</th>
<th>Culture filtrate (units/20 ml)</th>
<th>Mycelia (units/total mycelia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.6</td>
<td>0</td>
<td>25.1±5.59^2</td>
</tr>
<tr>
<td>2.6</td>
<td>0</td>
<td>176±28.3^2</td>
</tr>
<tr>
<td>3.6</td>
<td>0</td>
<td>*</td>
</tr>
</tbody>
</table>

Each value is the mean±SD (n=3).

1. Hydroxylase activity (units) was calculated on the basis of total mycelia recovered from a culture flask.

2. This activity was not detected when using heated mycelia (100°C, 5 min).

3. Mycelia were not collected because of autolysis.

**Hydroxylase activities of the mycelia and the culture filtrates incubated for different periods**

In Table 1, the conversion of daidzin and genistin into 8-OHD and 8-OHG, respectively, is presumed to have been responsible for the hydroxylase produced by *A. saitoi*. The microbial hydroxylation of various natural compounds has been reported by many investigators. With flavonoids, taxifolin (3’,4’,5,7-tetrahydroxyflavanonol) was transformed to dihydrodiosgenin (3’,4’,5,7,8-pentahydroxyflavanonol) by hydroxylase produced from a *Pseudomonas* sp. The *Micrococcus* and *Arthrobacter* species have recently been shown to transform 5-hydroxyisoflavones biochanin A and genistin to polyhydroxylated isoflavones by hydroxylation reactions at positions C-6 and C-8. However, no information on the hydroxylation reaction of isoflavone by *A. saitoi* has been reported.
In this experiment, the hydroxylase activities were determined by using genistein as an isoflavone substrate. The total hydroxylase units of the mycelia and the culture filtrates incubated for different periods are shown in Table 3. Hydroxylase activity was not detected in all the culture filtrates incubated for different periods. However, this activity appeared in the mycelia incubated for 1.6 d, which was the stage for starting sporulation, in addition to the new production of both 8-OHD and 8-OHG as shown in Fig. 4. The hydroxylase activity increased with the progress of sporulation. The total activity of the mycelia in a flask incubated for 2.6 d was about 7 times the activity after 1.6 d. These results correspond to the formation of 8-OHD and 8-OHG in Fig. 4. Using heated mycelia as enzyme material, no such conversion of genestein into 8-OHG was apparent (data not shown).

**Formation mechanism for the potent antioxidative isoflavones in soybeans fermented with A. saitoi**

The results of these present studies enable the formation process of 8-OHD and 8-OHG to be summarized as shown in the Scheme. Daidzin and genistein, which each have a β-glucosidic linkage, were gradually hydrolyzed into the corresponding aglycones daidzein and genistin, respectively, by β-glucosidase produced from the A. saitoi fermentation. This hydrolysis tended to proceed slowly during the stage of vegetative hypha growth because of its poor production of β-glucosidase. During this stage, no 8-OHD or 8-OHG was detectable. β-Glucosidase activities were greatly enhanced at the stage of sporulation. Therefore, daidzein and genistin were found to decrease and their aglycones to increase during this stage. The resulting daidzein and genistein were then hydroxylated to produce the potent antioxidants, 8-OHD and 8-OHG, respectively, by the hydroxylase produced from the A. saitoi fermentation during the stage of sporulation. Further research will be necessary to isolate and characterize both the β-glucosidase and the hydroxylase.

**Acknowledgments**

We are grateful to Bio’c Co. Ltd. and Ichibiki Co. Ltd. for generously providing the samples used in this study. We thank Ms. H. Ito for her technical assistance.

**References**


