Antiviral Activity of a Hot Water Extract of Black Soybean against a Human Respiratory Illness Virus

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Significant antiviral activity against respiratory illness viruses has been found in a hot-water extract of black soybean. This black soybean extract showed significant antiviral activity against human adenovirus type 1 and coxsackievirus B1 in a dose-dependent manner, while the hot-water extract from common yellowish soybean showed only weak activity. The antiviral activity could not be extracted from the black soybean by 70% aqueous ethanol, suggesting that saponin in the seed did not contribute to this activity. The antiviral activity was only recovered from cotyledons and not from seed coats with the hot water, showing that the activity was distributed in the cotyledons and that anthocyanins in the black soybean seed coats did not contribute to the antiviral activity. The antiviral compound(s) in the black soybean was partially purified by up to 166 times by a combination of gel filtration, reversed phase HPLC, and ion-exchange HPLC. The partially purified antiviral compound showed hydrophilic and anionic properties, and a maximum absorption at 260 nm, suggesting that this antiviral fraction may contain a phenyl group(s). On the other hand, an amino acid analysis with the acid hydrolyzate and a neutral sugar analysis showed that the antiviral compound from black soybean might not be a polypeptide or glycoconjugate bearing neutral sugar(s).

Key words: black soybean; hot-water extract; antiviral activity; respiratory illness virus; Glycine max

It has been reported that black soybean (kuromame in Japanese), which is a type of soybean (Glycine max (L.) Merrill) with a black seed coat,1) contained numerous bioactive compounds showing radical-scavenging activity,2–4) anti-tumor activity,4,5) and activity for improving the fluidity of the whole blood.6) Furthermore, an infusion (hot-water extract) of black soybean has been traditionally used as a folk medicine for treating pharyngolaryngeal symptoms in such acute respiratory diseases as sore throat, and for improving hoarseness (hoarse voice) and sputum expectoration. Since acute respiratory tract diseases are mainly caused by infection from viruses such as the adenovirus, influenza virus, and coxsackievirus,7) we have presumed that the pharmacological effect of the black soybean extract on the disease might be due to antiviral activity. Concerning the antiviral activity of soybean, it has been reported that soyasaponin from the common yellowish soybean inhibited the replication of the herpes simplex virus,8,9) human immuno-deficiency virus (HIV),8,10,11) cytomegalovirus,8) and influenza virus.8) However, the specific antiviral activity of the black soybean extract against...
respiratory illness viruses has not been previously investigated.

In this report, therefore, we describe for the first time the significant antiviral activity of a crude hot-water extract of black soybean against the adenovirus, coxsackievirus, and influenza virus, showing that the black soybean extract must have contained potent antiviral compound(s). Our result clearly shows that the putative antiviral compound(s) in black soybean would be a possible candidate for a highly effective medicine to treat infection by respiratory illness viruses.

Materials and Methods

Materials for the cell culture and chemicals. Phosphate-buffered saline (PBS) was purchased from Gibco (Gaithersburg, MD, U.S.A.), and Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Bovine serum albumin (fraction V) was purchased from Nacalai Tesque (Kyoto, Japan), and L-glutamine and fetal calf serum (FCS) were obtained from ICN Biomedicals (Aurora, Ohio, U.S.A.). Ribavirin, penicillin G, and streptomycin were purchased from ICN Biomedicals (Aurora, Ohio, U.S.A.). Ribavirin, penicillin G, and streptomycin were purchased from ICN Biomedicals (Aurora, Ohio, U.S.A.).

Viruses. Human adenovirus type 1 (Ad1), coxsackievirus B1 (CB1) and influenza A (H3N2) virus (FluV A) that were used for the in vitro antiviral assays were adapted and passaged more than three times in respective host cells. When a characteristic cytopathic effect (CPE) was apparent according to each viral infection, the infected cultures were frozen and thawed three times and then centrifuged (3,000 g, 10 min), before the supernatants were stored at −80°C until needed.

WST-1 assay. To evaluate whether the test sample bore cytotoxicity or cell-activation activity toward the host cells, the relative cell viability was measured as the mitochondrial NADH-dependent dehydrogenase activity with a cell counting kit, based on the WST-1 colorimetric assay. When a confluent culture had been formed on duplicated wells of 96-
well plates, the cells were further incubated with the black soybean sample dissolved in MEM containing 2% inactivated FCS (the maintenance medium) for 2 to 4 days at 37°C in 5% CO₂. The cells were gently washed twice with PBS, and then 100 μl of the same maintenance medium free from phenol red and 10 μl of 5 mM WST-1 were added to each well. After incubating for 2 hours at 37°C in 5% CO₂, the absorbance at 415 nm for a yellow-colored formazan dye reduced by cellular dehydrogenase, and at 600 nm for the background were measured by a microplate reader (MTP-120, Corona Electric, Tokyo, Japan). The difference in absorbance at A415 nm and A600 nm was used as the absorbance value. The cell viability is expressed as a percentage of this absorbance value obtained for the treated wells relative to that for the untreated control wells. The cytotoxic concentration (CC₅₀) is defined as the concentration of a sample that decreased the viability of uninfected cells to 50%.

In vitro antiviral activity assay. The antiviral activity of the black soybean extract against human respiratory illness viruses (human adenovirus type 1, coxsackievirus B1, and influenza virus A (H3N2)) was evaluated by using the cultured cell system. Briefly, serial two-fold dilutions of the sample were prepared in MEM containing 2% inactivated FCS for the anti-Ad1 assay and anti-CBI assay, and in MEM containing 1% bovine serum albumin (BSA) for the anti-FluV A assay. Simultaneously, serial 10-fold dilutions (10⁻⁷–10⁻⁵) corresponding to 10–100,000 TCID₅₀ (50% tissue culture infective dose) of the viral infective titer were prepared with the same medium. Virus dilutions were inoculated into confluent host cells in quadruplicate wells of 96-well plates and incubated for 1 h at 37°C in 5% CO₂. After removing the virus solution from each well, the well was washed twice with PBS. The sample solution (100 μl) was then immediately added to the well, and the cultures were further incubated under the conditions just described. When CPE in the virus-infected cells was observed microscopically, TCID₅₀ was determined by the method of Reed and Muench as a reciprocal of the lowest viral dilution that caused CPE in half of the total wells. The inhibitory effect (antiviral index) is expressed by the difference of log₁₀ TCID₅₀ between the control and treated cells. When this difference (antiviral index) was more than 1.0 and less than 1.5, the activity was rated as slightly active. When the index was more than 1.5 and less than 2.0, the activity was rated as active, and when the index was more than 2.0, the activity was rated as significantly active. As a positive control, ribavirin, a strong synthetic antiviral compound, was used to check the viral activity. The antiviral activity of the samples was statistically compared by using Student’s t-test.

Partial purification of the antiviral activity from the hot-water extract of black soybean. The hot-water extract of black soybean was centrifuged (10,000 g, 15 min), and the resulting supernatant was filtered with a 10-kDa molecular weight cut-off (MWCO) membrane (Ultrafree-1 ultrafilter unit with a Biomax-5 membrane; Millipore Japan, Tokyo, Japan) and then with a 5-kDa MWCO membrane (Ultrafree-0.5 ultrafilter unit with a Biomax-10 membrane; Millipore Japan, Tokyo, Japan). Since the antiviral activity against Ad1 was recovered in the upper layer of the 5-kDa MWCO, the concentrated extract was put on a Sephadex G-25 column (1.8 × 180 cm) that had been equilibrated with 0.1 N NH₄OH. The eluate was monitored by measuring the absorbance at 280 nm and by the phenol-sulfuric acid method. The fraction containing anti-Ad1 activity was dissolved in a small amount of 0.02% trifluoroacetic acid (TFA) and applied to reversed phase HPLC (RP-HPLC) in a Poros R2 column (4.6 × 100 mm, Perpective) that had been equilibrated with 0.02% TFA. The column was washed with the same solvent, and then the bound compounds were eluted by a linear gradient of acetonitrile from 0 to 80% at a flow rate of 1.5 ml/min. The run-through fraction containing the anti-Ad1 activity was concentrated to dryness and then dissolved in a 25 mM Tris-HCl (pH 8.5) buffer containing 50 mM NaCl. The antiviral fraction obtained by RP-HPLC was loaded into a Shodex IEC QA-825 column (0.8 × 7.5 cm, Showa Denko Co.) that had been equilibrated with the same buffer. After washing the column, the adsorbed compounds (acidic compounds) were eluted by a linear gradient of NaCl concentration from 50 mM to 250 mM at a flow rate of 1.0 ml/min. The eluate was monitored by measuring the absorbance at 280 nm. When the antiviral activity was measured with the partially purified antiviral compound, we also used the fluorescamine method to quantify a small amount of the sample, assuming that the antiviral compound would bear an amino group(s). BSA was used as a standard compound with the fluorescamine method.

Amino acid analysis and carbohydrate analysis. An amino acid analysis of the black soybean antiviral compound was conducted by the method of Bidlingmeyer et al. after acid hydrolysis with 5.7 N HCl containing 0.02% 2-mercaptoethanol.

Results and Discussion

Anti-Ad1 activity of black soybean

The adenovirus, which causes various symptoms of respiratory illness such as laryngitis and upper respiratory inflammation, infects the epithelial cells or mucus membrane cells of the respiratory tract, conjunctiva, cornea, and other organs. As a preliminary experiment to confirm the presence of sig-
significant antiviral activity in black soybean, the inhibitory effects of hot-water extracts from black soybean and common yellowish soybean on the Ad1 replication in HeLa cells were compared. As shown in Fig. 1-A, the hot-water extract from black soybean demonstrated dose-dependent inhibitory activity against Ad1 replication; antiviral activity (the antiviral index was about 1.5) was observed at 1.5 mg/ml, and significant activity (the antiviral index was 2.0) at 3.5 mg/ml. On the other hand, the hot-water extract from the common yellowish soybean showed activity (the antiviral index = 1.5) at about 6.0 mg/ml. This result shows that the hot water extract of black soybean carried higher antiviral activity than that of the common yellowish soybean, suggesting that black soybean contained characteristic antiviral compound(s). Similar antiviral activity of the black soybean extract against human adenovirus type 2 was also observed, although the extract showed little activity against human adenovirus types 3 and 7 (data not shown).

Interestingly, the 70% aqueous ethanol extract of black soybean demonstrated only negligible activity (shown in Fig. 1-A). The antiviral activity of soyasaponin against the herpes simplex virus, human immunodeficiency virus, human cytomegalovirus, and FluA has been reported.\textsuperscript{8-11} Since it has been reported that soyasaponin was recovered in 70% aqueous ethanol, we presumed that the antiviral activity of the hot-water extract of black soybean against Ad1 would have come from compound(s) other than soyasaponin. As shown in Fig. 1-A, the antiviral activity against Ad1 was recovered by hot-water extraction from the 70% aqueous ethanol-treated black soybean seeds, showing that the antiviral activity against Ad1 must have come from a water-soluble or hydrophilic compound(s). These observations indicated that hot water would be an appropriate solvent for extracting the antiviral component in black soybean, and the traditional use of hot water for extracting a black soybean infusion as a folk medicine seemed to be reasonable.

To clarify the distribution of the antiviral activity against Ad1 in the black soybean seeds, we prepared two extracts; one was from seed coats and the other from cotyledons. The extract from the seed coat showed cytotoxicity at a concentration of more than 0.6 mg/ml (data not shown). It therefore seems that the apparent antiviral activity of the seed coat extract decreased (Fig. 1-B). On the other hand, the extract from the cotyledons showed much stronger activity against Ad1 replication than that from the seed coats, suggesting that the antiviral activity was distributed in black soybean cotyledons. We also found that cyanidin-3-glucoside, one of the major anthocyanins in the black soybean seed coat,\textsuperscript{19} did not inhibit the replication of Ad1 (data not shown).

Antiviral activity of black soybean against other respiratory viruses

The adenovirus is epidemiologically detectable throughout the year. On the contrary, the coxsackievirus causes aseptic meningitis or herpangina mainly in the summer season, and contagious infec-
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The influenza virus causes severe acute respiratory disease in the winter season. As already described, the hot-water extract of black soybean was found to have antiviral activity against human adenovirus type 1. We also examined the antiviral activity of the black soybean extract against the coxsackievirus (CB1) and influenza virus (FluV A). The hot-water extract of black soybean showed an inhibitory effect against CB1 replication at approximately 2.6 mg/ml, and a significant effect at approximately 4.7 mg/ml. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a well-known antiviral agent possessing a broad-spectrum nucleoside, showed anti-CB1 activity at approximately 0.25 mg/ml, and significant activity at approximately 0.42 mg/ml (Fig. 2-A). The black soybean extract and ribavirin also showed anti-CB1 activity toward FL cells used as the host cell (Fig. 2-B); 7.0 mg/ml of the black soybean extract and 0.28 mg/ml of ribavirin were required to demonstrate antiviral activity (the antiviral index was 1.5). As shown in Fig. 3, although the hot-water extract of black soybean showed much weaker anti-FluV A activity than ribavirin, the 10 mg/ml dose of the black soybean extract showed antiviral activity (the antiviral index was 1.5). These results suggest that the antiviral activity of the hot-water extract from black soybean had a broad spectrum against the replication of respiratory viruses in cultured cells.

Cytotoxicity and cell activation of the hot-water extract from black soybean

The cytotoxicity and cell activation activity of the hot-water extract from black soybean toward HeLa cells, FL cells, MDCK cells, and Vero cells were assayed by the WST-1 test. The CC50 values for each type of cell are shown in Table 1. The black soybean extract at the concentration giving antiviral activity (less than 10 mg/ml) showed no cytotoxicity toward HeLa cells, FL cells, or MDCK cells. Therefore, the antiviral activity against Ad1, CB1, and FluV A must have been due to the putative activity against the replication of these viruses rather than to the cytotoxicity to the cultured cells. The hot-water-extract of black soybean and ribavirin showed cytotoxicity toward Vero cells at the respective concentrations of less than 5 mg/ml and 0.2 mg/ml, suggesting that the
Vero cell was not appropriate for an assay of antiviral activity. A substantial increase in absorbance in the WST-1 test was detected when HeLa cells were incubated with the black soybean hot-water extract in a concentration range of 0.1–5 mg/ml (Fig. 4), indicating that the extract would activate mitochondrial NADH-dependent dehydrogenase in the cells or induce cell growth. On the other hand, at a concentration of 10 mg/ml, although the extract showed slight cytotoxicity, the relevant antiviral activity still remained, suggesting that the antiviral activity might overwhelm the cytotoxicity. In fact, the apparent antiviral activity decreased due to the cytotoxicity at a concentration of more than 20 mg/ml (data not shown), suggesting that the extract might activate the activity of HeLa cells but not their growth. These results indicate that the hot-water extract of black soybean could activate the cells to prevent viral replication.

Partial purification of the antiviral compound in the hot-water extract of black soybean

The hot-water extract of black soybean (143.9 g) was filtered through the 10-kDa MWCO membrane, and anti-Ad1 activity was exclusively recovered in the filtrate. The filtrate was further filtered by the 5-kDa MWCO membrane. No anti-Ad1 activity was detected in the filtrate, suggesting that the molecular mass of the antiviral compound(s) would be less than 10 kDa. The antiviral active fraction (100 mg) obtained by ultrafiltration was dissolved in 0.1 N NH₄OH and applied to a Sephadex G-25 column, the eluate being monitored by the absorbance at 280 nm. As shown in Fig. 5-A, three fractions were obtained, and the antiviral activity of each was assayed. The second fraction showed the strongest anti-Ad1 activity, this active fraction being negative by the phenol-sulfuric acid method, suggesting that the putative antiviral compound did not contain neutral sugars. The active fraction obtained by gel filtration was further purified by RP-HPLC in a Poros R2 column. The total anti-Ad1 activity was recovered in the runthrough fraction, suggesting the antiviral compound to be a hydrophilic organic compound(s) (Fig. 5-B). The active fraction was concentrated to dryness, and the residue was dissolved in 5 ml of a 25 mm Tris-HCl buffer (pH 8.5) containing 50 mm NaCl. After centrifugation, the supernatant was applied to a Shodex IEC QA-825 column, and the antiviral compound was eluted by increasing NaCl concentration in the same buffer. As shown in Fig. 5-C, six fractions (F-I–F-VI) were obtained, and the antiviral activity in each fraction was assayed. The relevant antiviral activity was detected in F-IV, suggesting the antiviral compound(s) to have hydrophilic and anionic properties.

The specific antiviral activity was assayed by using the antiviral active fractions obtained in each purification step. To quantify the amount of the antiviral compound, we used the fluorescamine method. We assumed that the antiviral compound might be a

### Table 1. Cytotoxicity of the Hot-water Extract from Black Soybean

<table>
<thead>
<tr>
<th>Host cell</th>
<th>Adapted virus</th>
<th>CC₅₀ (mg/ml)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>Ad1, CB1</td>
<td>15.3</td>
</tr>
<tr>
<td>FL</td>
<td>CB1</td>
<td>22.3</td>
</tr>
<tr>
<td>Vero</td>
<td>CB1, echovirus 1</td>
<td>3.5</td>
</tr>
<tr>
<td>MDCK</td>
<td>FluV A</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Cytotoxicity was assayed by the WST-1 test.

¹ CC₅₀: 50% cytotoxic concentration.
Fig. 5. Purification Steps for the Antiviral Compound from the Hot-water Extract from Black Soybean.

A: Gel-filtration of the crude extract from black soybean in the Sephadex G-25 column (1.8 × 180 cm). The hot-water extract of black soybean was centrifuged and filtered through a 10-kDa MWCO membrane and then through a 5-kDa MWCO membrane. The extract recovered from the upper layer of the 5-kDa MWCO membrane containing anti-Ad1 activity was concentrated and put on a Sephadex G-25 column (1.8 × 180 cm) that had been equilibrated with 0.1N NH₄OH. The antiviral activity was exclusively detected in the second fraction, designated as F-II. B: RP-HPLC of the anti-Ad1 fraction obtained in A. F-II obtained in A was evaporated to dryness. The resulting residue was dissolved in a small amount of 0.02% trifluoroacetic acid (TFA) and applied to a Poros R2 column (0.46 × 10 cm). The bound compounds were eluted by an increasing concentration of acetonitrile in the solvent. The antiviral activity was exclusively detected in the run-through fraction indicated by a bar. C: Ion-exchange HPLC of the anti-Ad1 fraction obtained in B. The run-through fraction obtained in B was concentrated to dryness, and the resulting residue was dissolved in a 25 mM Tris-HCl buffer (pH 8.5) containing 50 mM NaCl. The sample was applied to a Shodex IEC QA-825 column (0.8 × 7.5 cm) that had been equilibrated with the same buffer. After washing the column, the bound compounds were eluted by an increasing concentration of NaCl. The relevant antiviral activity was recovered in F-IV.

Fig. 6. Comparison of the Activities of the Antiviral Fractions Obtained in Each Purification Step.

The amino acid analysis of the acid hydrolyzate of the partially purified antiviral active fraction obtained by RP-HPLC showed that the putative black soybean antiviral compound(s) did not contain any standard amino acid in a relevant amount. However, the absorption spectrum of the partially purified antiviral active fragment obtained by ion-exchange HPLC showed a maximum absorbance at 260 nm, suggesting that the antiviral compound may have contained the phenyl group(s). Although some antiviral compounds having aromatic groups (synthetic antiviral agents²²,²³) and polyphenolic compounds²⁴–²⁷) isolated

polypeptide such as soyacystatin²⁰ or oryzacystatin,²¹ since the antiviral activity could always be monitored by the absorbance at 280 nm. We used bovine serum albumin (BSA) as a standard polypeptide for the fluorescamine method; therefore, the concentration of the antiviral compound is expressed as the amount of BSA for the sake of convenience. As shown in Fig. 6, the specific activity significantly increased as the purification proceeded, although we could not use F-IV for ion-exchange HPLC due to the small amount. The activity of the run-through fraction obtained by RP-HPLC (about 9 mg/ml was needed to show an antiviral index of 2) was about 166 times higher than that of the crude extract (about 1500 mg/ml was needed to show an antiviral index of 2).

The amino acid analysis of the acid hydrolyzate of the partially purified antiviral active fraction obtained by RP-HPLC showed that the putative black soybean antiviral compound(s) did not contain any standard amino acid in a relevant amount. However, the absorption spectrum of the partially purified antiviral active fragment obtained by ion-exchange HPLC showed a maximum absorbance at 260 nm, suggesting that the antiviral compound may have contained the phenyl group(s). Although some antiviral compounds having aromatic groups (synthetic antiviral agents²²,²³) and polyphenolic compounds²⁴–²⁷) isolated

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from natural products) have been reported, the molecular masses of these compounds are less than 1 kDa. Therefore, the antiviral compound(s) from black soybean against the adenovirus, coxsackievirus, and influenza virus, showing that the black soybean extract must have contained potent antiviral compound(s). Our results clearly show that the putative antiviral compound(s) in black soybean would be a possible candidate for a highly effective medicine against respiratory illness viruses. Since the structural identification of the antiviral compound(s) could not be completed due to small amount of the purified sample, purification of a larger amount and a detailed structural analysis are in progress to elucidate the molecular mechanism for the antiviral activity.

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