

Cloning and Characterization of Saponin Hydrolases from *Aspergillus oryzae* and *Eupenicillium brefeldianum*

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Received June 14, 2005; Accepted July 22, 2005

We purified saponin hydrolases from *Aspergillus oryzae* PF1224 and *Eupenicillium brefeldianum* PF1226. It was confirmed that the enzymes from *A. oryzae* PF1224 (Sda1) and *E. brefeldianum* PF1226 (Sde1) are glycoproteins with molecular masses of 82 and 90 kDa respectively. The deduced amino acid sequences of each enzyme from the cloned genes (*sda1* or *sde1*) showed approximately 50% homology with that of the saponin hydrolase Sdn1 from *Neocosmospora vasinfecta* var. *vasinfecta* PF1225 (DDBJ accession no. AB110615). When *sda1* and *sde1* were expressed in the host *Trichoderma viride* under the control of the cellobiohydrolase I gene promoter, recombinant proteins were secreted with molecular masses of 77 and 67 kDa respectively. These recombinant enzymes hydrolyzed soyasaponin I to soyasapogenol B and triose, and its substrate specificities for glycosides were similar to that of Sdn1, but the specific activities of these enzymes were lower than that of Sdn1.

Key words: soyasaponins; soyasapogenol B; *Aspergillus oryzae*; *Eupenicillium brefeldianum*

Saponins, which are glycosides of triterpenoids or steroids, are widely distributed in the plant kingdom. Among them, soyasaponins are triterpene glycosides with aglycones of oleanene skeleton like soyasapogenols.¹⁾ These molecules are found in various leguminous plants, e.g., soybean, adzuki bean, peanut, and alfalfa.^{2,3)} Since soyasaponins and soyasapogenols have various physiological effects including hepatoprotective,^{4–7)} anticarcinogenic,^{8–11)} antiviral,¹²⁾ and anti-inflammatory¹³⁾ activities, it is necessary to establish efficient methods to remove or modify their carbohydrates.

We screened *Neocosmospora vasinfecta* var. *vasinfecta* PF1225, which was isolated from soil samples and selected on the basis of its ability to produce soyasapogenol B from soybean saponins added to the culture medium.¹⁴⁾ In addition, saponin hydrolase was purified from this fungus, and the gene encoding it was isolated based on its amino acid sequences. We confirmed that the saponin hydrolase Sdn1 is a monomeric glycoprotein

with a molecular mass of 77 kDa.¹⁵⁾ The molecular mass of Sdn1 was not the same as that of soybean saponin hydrolase, a hetero tetramer with a molecular mass of 158 kDa purified from saprophyte, *Aspergillus oryzae* KO-2.¹⁶⁾ The deduced amino acid sequence showed no homology with any of the glycoside hydrolases reported previously. And the only sequence showed approximately 50% similarity with the expression sequence tag (EST) sequence derived from vascular wilt pathogen, *Verticillium dahliae*.¹⁷⁾ From these results, we speculated that this enzyme system might be a phytopathogen specifically functioning at the time of infecting plants, and that it has the potential to promote the establishment of infection by detoxifying saponins.

By screening of soyasapogenol B-producing activity, several kinds of filamentous fungi have been isolated that belong to the saprophytes.^{14,18)} Hence, in the present study, we investigated the cloning and characterization of enzymes with soyasapogenol B-producing enzymatic activity, which were derived from the saprophytes *A. oryzae* PF1224 and *Eupenicillium brefeldianum* PF1226 isolated from soil samples. We clarified the primary structures of the genes encoding these enzymes, and compared them with enzymes derived from related phytopathogen strain.

Materials and Methods

Chemicals. Soybean extract I (content of soybean saponin, 32%), used as soybean saponin, was purchased from Tokiwa Phytochemical (Japan). Authentic soyasapogenol B was prepared as described previously.¹⁴⁾ Glycyrrhizic acid monoammonium salt *n*-hydrate (glycyrrhizin), glycyrrhetic acid 3-*O*-glucuronide, glycyrrhetic acid, 4-methylumbelliferyl- β -D-glucuronide (4MU-glucuronide), and 4-methylumbelliferone were purchased from Wako Pure Chemical Industries (Osaka, Japan). Soyasaponins I, II, and V were prepared as described previously.¹⁵⁾ The structures of soyasaponins and glycyrrhizins are summarized in Fig. 1.

Strains and media. *A. oryzae* PF1224 and *E. brefel-*

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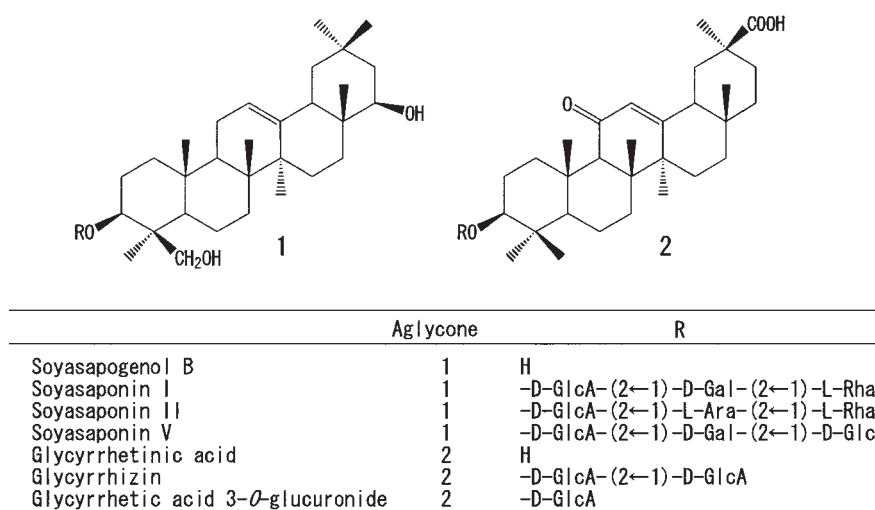


Fig. 1. Structures of Soyasaponins and Glycyrrhizinic Acids.

GlcA, glucuronic acid; Gal, galactose; Rha, rhamnose; Ara, arabinose; Glc, glucose.

dianum PF1226, stock strains in our collection, were used for purification of soybean saponin hydrolases. These strains were maintained on PDA medium (Becton Dickinson, Sparks, MD) and were cultured in TS medium (20 g soluble starch, 10 g glucose, 5 g polypepton, 6 g wheat germ, 3 g yeast extract, and 2 g CaCO_3 in 1 liter, pH 7.0) and MY medium (40 g malt extract, 20 g yeast extract, 2 g KH_2PO_4 , 2 g $(\text{NH}_4)_2\text{SO}_4$, 0.3 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 10 g soybean saponin in 1 liter, pH 7.0) for induction of soyasapogenol B-producing enzyme.¹⁵⁾ *Trichoderma viride* strain 2 was used as a host for recombinant enzyme production, and transformants that expressed saponin hydrolases were cultured in P medium to obtain recombinant enzymes.¹⁹⁾

Measurement of enzyme activities. Enzymatic activity was measured by quantitative analysis of soyasapogenol B from a reaction mixture consisting of 1% soybean saponin, 0.1 M sodium phosphate (pH 5.8), and diluted enzyme fraction.¹⁵⁾ One unit of activity of each enzyme was defined as the amount of enzyme that produces 1 nmol of aglycone per min from the substrate.

Purification of a soyasapogenol B-producing enzyme from *A. oryzae* PF1224. *A. oryzae* PF1224 was cultured in MY medium for 3 d, and the culture supernatant was obtained by centrifugation. Ammonium sulfate was added to the crude enzyme fraction to 70% saturation, the solution was centrifuged, and the precipitate was dissolved in 0.1 M sodium acetate (pH 5.8) and 1 M ammonium sulfate. This solution was applied to a Toyopearl Butyl-650S (Tosoh, Tokyo) column. Soyasapogenol B-producing activity was eluted at 1 to 0.5 M ammonium sulfate concentration by 1 to 0 M linear gradient elution in 0.1 M sodium phosphate (pH 5.8). This active fraction was concentrated and then applied to a Resource PHE (Amersham Biosciences, Piscat-

away, NJ) column, and eluted with 1 M ammonium sulfate in 50 mM Tris-HCl (pH 7.5). This eluate was applied to a Superdex 200 pg (Amersham Biosciences) column, and eluted with 25 mM sodium phosphate (pH 5.8) and 0.15 M sodium chloride. Finally, the active fraction was eluted at a molecular mass of 90 kDa using catalase (232,000 Da), aldolase (158,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), and chymotrypsinogen A (25,000 Da) as protein standards.

Purification of a soyasapogenol B-producing enzyme from *E. brefeldianum* PF1226. *E. brefeldianum* PF1226 was cultured in MY medium, except that the culturing time was set at 7 d. The culture supernatant was prepared by centrifugation and the crude protein fraction was precipitated with ammonium sulfate in 70% saturation. The precipitate was dissolved in 50 mM Tris-HCl (pH 7.5) and 1 M ammonium sulfate and applied to a Toyopearl Butyl-650S column. Chromatography was performed with a 1 to 0 M ammonium sulfate linear gradient in 50 mM Tris-HCl (pH 7.5), and the active fraction was eluted with 0.7 to 0.5 M. Next, this pool was concentrated and applied to a Resource PHE column, and the active fraction was eluted with 1 to 0.3 M ammonium sulfate. After concentration and desalting, this fraction was applied to a Resource Q (Amersham Biosciences) column to collect the fraction eluted with 20 mM sodium phosphate (pH 7.0). Finally, this fraction was applied to a Superdex 200 pg column and the active fraction was eluted with a molecular mass of 80 kDa.

Cloning of saponin hydrolase genes. The saponin hydrolase derived from *A. oryzae* PF1224 (Sda1) was subjected to SDS-PAGE and blotted onto a PVDF membrane (Immobilon P^{SQ}, Millipore, Bedford, MA). A blot was applied to a Model 492 protein sequencer (Applied Biosystems, Foster City, CA) to analyze the N-

terminal amino acid sequence. According to the in-gel digestion method with trypsin,²⁰ four peptides were separated by high-pressure liquid chromatography (HPLC), and the N-terminal amino acid sequences were analyzed. From these sequences, a mix primer (AP24.00s; CARTTYAAYCCIGCNCC, AP38.05a1; AARTCNGARAACCARTC) was synthesized and used for amplification by polymerase chain reaction (PCR) with genomic DNA as a template. The PCR conditions were set as described previously.¹⁵ Genomic DNA was digested with *Hind*III and separated with agarose gel electrophoresis, and approximately 5-kbp fragments were recovered and ligated with pUC18, and then transformed to *E. coli* to construct the partial *Hind*III plasmid library. The saponin hydrolase gene was screened from this partial genomic library using PCR products as a probe.

The amino acid sequence of the saponin hydrolase derived from *E. brefeldianum* PF1226 (*Sde1*) was analyzed as described for *Sda1* to obtain the N-terminal amino acid sequence and three internal peptide sequences. From these sequences, a mix primer (EPNs; CCICARCCNGARCCNAT, EP20.73a1; CTRAAGC-NGGRTCNGC) was synthesized and used for PCR amplification. Then the saponin hydrolase gene from *E. brefeldianum* PF1226 was screened using the PCR product as a probe for the genomic library prepared by EMBL3.

Plasmids for the expression of saponin hydrolase genes in *T. viride*. PCR was carried out using a cloned gene as the template and primers for expression of *Trichoderma* (primer sets for *sda1*: GGGAGGCCT-GCGCATCATGCATGTTGTCTCGCAAGTACCAC and GGGCTCGAGTACCTCAAGTCCCATTGCGCGGCT-GC, primer sets for *sde1*: GGGAGTACTGCGCATC-ATGCGTTGGAGTTTCTCTGTGTC and GGGCTCGAG-TACCTCATGCACCATTTGAGCGGCTGGTGG). The amplified coding regions were digested with *Stu*I and *Xho*I for *sda1*, and *Sca*I and *Xho*I for *sde1*, and were ligated to pCB1-M2¹⁹ that had been digested with *Stu*I and *Xho*I. As results, plasmids pCB-SDAe and pCB-SDEe were constructed.

Transformation of *T. viride*. pCB-SDAe or pCB-SDEe was transformed in *T. viride* by co-transformation with plasmid pPYR4 containing the *pyr4* gene from *Neurospora crassa*.¹⁵ The transformation procedure was as described previously.¹⁵

Purification of the recombinant enzymes. *T. viride* SDA30/pCB-SDAe was cultured in P medium,¹⁹ and the culture supernatant was obtained by centrifugation. Then ammonium sulfate was dissolved in the supernatant to 60% saturation, and the precipitate was collected by centrifugation. This fraction was applied to a Toyopearl Butyl-650S column and eluted with 0.9 to 0.2 M ammonium sulfate in 0.1 M sodium phosphate

(pH 5.8). After concentration and desalting, it was applied to a Resource Q column to collect the non-adsorbed fraction and the fraction eluted with 0 to 0.08 M sodium chloride in 50 mM Tris-HCl (pH 7.5). After concentration, this fraction was applied to a Superdex 200 pg column, and the active fraction was collected by elution with 50 mM sodium phosphate (pH 7.0) and 0.15 M sodium chloride.

T. viride SDE26/pCB-SDEe was cultured the same way as *T. viride* SDA30/pCB-SDAe, and the culture supernatant was obtained by centrifugation. Precipitate from a 40% saturated ammonium sulfate fraction of the supernatant was obtained by centrifugation. This fraction was applied to a Toyopearl Butyl-650S column to collect the fraction eluted with 0.2 to 0 M ammonium sulfate in 0.1 M sodium phosphate (pH 5.8). After concentration and desalting, it was applied to a Resource Q column to collect the fraction eluted with 0 to 0.1 M sodium chloride in 50 mM Tris-HCl (pH 7.5). After concentration, this fraction was applied to a Superdex 200 pg column to collect the active fraction, which was eluted with 50 mM sodium phosphate (pH 7.0) and 0.15 M sodium chloride.

Protein determination, SDS-PAGE, and deglycosylation of the purified enzymes. The protein content of the purified proteins was determined with a protein assay kit (Bio-Rad, Hercules, CA) using bovine gamma globulin as the standard. SDS-PAGE was carried out using SDS-PAGE mini (Tefco, Tokyo). SYPRO ruby gel stain (Molecular Probes, Eugene, OR) was used for SDS-PAGE gel staining, and Molecular Imager FX (Bio-Rad) was used for detection. Calculation of molecular masses of SDS-PAGE was done with the Quantity One program (Bio-Rad). Deglycosylation of purified enzymes was done with the *N*-glycosidase F deglycosylation kit (Roche Diagnostics, Mannheim, Germany).

Nucleotide sequence accession number. The DNA sequences of *sda1* and *sde1* are deposited under accession nos. AB182941 and AB182942 respectively in the DDBJ, EMBL, and GenBank databases.

Results

*Purification of soyasapogenol B-producing enzymes from *A. oryzae* PF1224 and *E. brefeldianum* PF1226*

A. oryzae PF1224 or *E. brefeldianum* PF1226 was cultured in MY medium with or without soybean saponin. Soyasapogenol B-producing activity was detected only in the supernatant from cultures containing soybean saponin. Hence, it was confirmed that these activities of both fungi are induced by soybean saponin, like that of saponin hydrolase *Sdn1* of *N. vasinfecta* var. *vasinfecta*.¹⁵

The soyasapogenol B-producing enzymatic activity of the culture supernatant of the 3d culture of *A. oryzae* PF1224 was 0.83 units/ml, lower than that of *Sdn1*

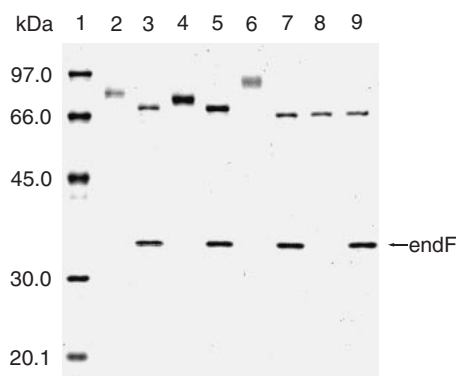


Fig. 2. SDS-PAGE Analysis of Purified Saponin Hydrolases.

Purified saponin hydrolases treated or not treated with *N*-glycosidase F were subjected to SDS-PAGE, stained with SYPRO ruby, and detected with Molecular Imager FX. An arrow indicates *N*-glycosidase F. Lane 1, molecular mass markers: phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa). Lane 2, saponin hydrolase purified from *A. oryzae* PF1224. Lane 3, *N*-glycosidase F treated saponin hydrolase purified from *A. oryzae* PF1224. Lane 4, saponin hydrolase purified from *T. viride* SDA30/pCB-SDAe. Lane 5, *N*-glycosidase F treated saponin hydrolase purified from *T. viride* SDA30/pCB-SDAe. Lane 6, saponin hydrolase purified from *E. brefeldianum* PF1226. Lane 7, *N*-glycosidase F treated saponin hydrolase purified from *E. brefeldianum* PF1226. Lane 8, saponin hydrolase purified from *T. viride* SDE26/pCB-SDEe. Lane 9, *N*-glycosidase F treated saponin hydrolase purified from *T. viride* SDE26/pCB-SDEe.

(9.1 units/ml, 3d culture).¹⁵⁾ No enzymatic activity of culture supernatants from *E. brefeldianum* PF1226 in 3d culture was detected, for the 7d culture it was 4.47 units/ml. The soyasapogenol B-producing enzymes of *A. oryzae* PF1224 (Sda1) and *E. brefeldianum* PF1226 (Sde1) were purified from supernatant of the soybean saponin inducing culture, proteins with a molecular masses of approximately 90 and 80 kDa respectively, by gel filtration. On the other hand, the molecular masses of Sda1 and Sde1 by SDS-PAGE were approximately 82 and 90 kDa respectively. Therefore, these enzymes were indicated to be monomers (Fig. 2, lanes 2 and 6).

Cloning of the genes encoding soyasapogenol B-producing enzymes

Five amino acid sequences (N-terminal, QQILKPPV-PEPI; peptide fragments digested with trypsin; Atrp23.67, LYNPDSPQPISAK; Atrp24.00, LQFN-PAPK; Atrp24.65, GEVSGSASVSIHD; Atrp38.05, VDWFSDLTSTGQVTGSK) were obtained from the N-terminal and peptides digested with trypsin of purified Sda1. These amino acid sequences showed similarity with Sdn1. Degenerate primers were designed based on these amino acid sequences, and PCR was carried out using the genomic DNA of *A. oryzae* PF1224 as a template. In this way, a fragment of approximately 1-kbp was specifically amplified using the primers corresponding to Atrp24.00; LQFNPAPK and

Atrp38.05; VDWFSDLTSTGQVTGASK. Southern blot analysis of genomic DNA was performed with PCR products as a probe, and a 5-kbp *Hind*III fragment was hybridized. Thus, using this PCR product as a probe, the partial genome library prepared by a *Hind*III fragment of approximately 5-kbp in pUC18 was screened and one positive clone was obtained. Analysis of the DNA sequence of a 5-kbp *Hind*III fragment obtained from the positive clone revealed that the gene, which had a coding region of 1,902 bp, consisted of 605 amino acids with a calculated molecular mass of 65,252 Da, and with a 28 amino acid secretion signal sequence upstream of mature N-terminal. Since the deduced amino acid sequence was included in all of the sequences determined from the peptide fragments, this gene was thought to encode Sda1 and was named *sda1*.

Four amino acid sequences (N-terminal, STTPAPPQ-PEPIEVV; peptide fragments digested with trypsin; Etrp20.73, ADPAFSPDGTR; Etrp34.21, LHPDD-THMGWSSF; Etrp36.26, GFSGAGDEILYIGSTR) were obtained from the N-terminal and peptides were digested with trypsin of purified Sde1. These amino acid sequences showed similarity with Sda1 and Sdn1. When degenerate primers were designed from these amino acid sequences for PCR, a fragment of 1.1-kbp was amplified with primers corresponding to the N-terminal: STTPAPPQPEPIEVV and Etrp20.73; ADPAFSPDGTR. Using this PCR product as a probe, the genomic DNA library prepared in EMBL3 was screened and a clone retaining a *Xho*I fragment of 6-kbp was obtained. This size for the *Xho*I fragment agreed with the result of genomic Southern blot analysis of *E. brefeldianum* PF1226. Analysis of the DNA sequence of this fragment revealed that a gene which had a coding region of 1,857 bp encoded a 601 amino acid mature protein with a molecular mass of 64,874 Da, with a 17 amino acid secretion signal sequence. Since the deduced amino acid sequence included all of the sequences determined from the peptide fragments, this gene was shown to encode Sde1 and was named *sde1*.

Because the molecular masses of the purified enzymes were higher than those calculated from the deduced amino acid sequences, it was suggested that the enzymes had undergone saccharification. The amino acid sequences of the saponin hydrolases contained 9 and 10 typical N-linked glycosylation sites for Sda1 and Sde1 respectively. The molecular masses of Sda1 and Sde1 were changed to 71 and 66 kDa respectively (Fig. 2, lanes 3 and 7) by treatment with *N*-glycosidase F. Sde1 was modified with asparagine link glycosylation, whereas the molecular mass of deglycosylated protein corresponded to the calculated molecular mass from the deduced amino acid sequence. On the other hand, the molecular mass of deglycosylated Sda1 was approximately 5,000 Da higher than that of the deduced amino acid sequence. Hence, Sda1 was thought to have glycosylated not only the asparagine link but also the serine/threonine link.

Plasmids pCB-SDAe and pCB-SDEe were constructed so that the coding region of *sdal* and *sdel* was controlled by the cellobiohydrazinase I (*cbhl*) gene promoter from *T. viride*. Each was co-transformed with pPYR4 to create a uracil dependent complementary plasmid.¹⁵ Twenty-four and 17 pCB-SDAe and pCB-SDEe transformants were obtained per microgram of DNA respectively. When these transformants were cultured under *cbhl* inducing conditions, half of the transformants of each plasmid had saponin hydrolase activity in the culture supernatants. When these culture supernatants were subjected to SDS-PAGE, the pCB-SDAe and pCB-SDEe transformants showed recombinant protein bands at approximately 77 and 67 kDa respectively. The highest activities of supernatants from a 4d culture of pCB-SDAe and pCB-SDEe transform-

ants (*T. viride* SDA30/pCB-SDAe and SDE26/pCB-SDEe) were 1658 and 3745 units/ml respectively. Hence, these recombinant proteins were purified and compared with respect to substrate specificity.

Recombinant Sda1 and Sde1 were purified from the culture supernatants of *T. viride* SDA30/pCB-SDAe and *T. viride* SDE26/pCB-SDEe, indicating molecular masses of 77 and 67 kDa by SDS-PAGE respectively (Fig. 2, lanes 4 and 8). On SDS-PAGE, the molecular masses of these recombinant saponin hydrolases were different from those of the enzymes purified from *A. oryzae* PF1224 and *E. breifeldianum* PF1226. Therefore glycoside chains were removed using *N*-glycosidase F and compared again. The molecular masses of *N*-glycosidase F treated recombinant Sda1 and Sde1 decreased to 71 and 66 kDa respectively. After removal of the asparagine linked glycoside chains, the molecular weights of the two enzymes were identical as between the wild type and the recombinant (Fig. 2, lanes 5 and 9).

Enzymatic properties of saponin hydrolases

The activity profiles of the saponin hydrolases were compared by determining the amount of soyasapogenol B released from soybean extract I as the substrate. The activities of Sda1, Sde1, recombinant Sda1, and recombinant Sde1 were 0.59, 1.36, 0.67, and 1.42 units/ μ g protein respectively. The optimum pH values at 37 °C were 5.2, 5.4, 5.2, and 5.6 for Sda1, Sde1, recombinant Sda1, and recombinant Sde1 respectively. The optimum temperature at pH 5.8 was 50 °C for Sda1 and recombinant Sda1, and 45 °C for Sde1 and recombinant Sde1. From these results, it was concluded that the recombinant saponin hydrolases are closely similar to the wild types with respect to their activity profiles, despite having different modifications of their glycoside chains. Hence the substrate specificities of the saponin hydrolases were compared with the recombinant enzymes.

When the activity of each recombinant saponin hydrolase was measured using purified soyasaponins as the substrate, Sda1 showed the lowest hydrolyzing activity among the three enzymes, and its substrate specificity also tended to be lower. The specific activity of Sde1 was higher than that of Sda1, but lower than that of Sdn1. Sde1 had the lowest glycyrrhizin hydrolyzing activity. All of the saponin hydrolases showed a similar relative soyasaponin hydrolyzing activity: soyasaponin I > soyasaponin II $\gg$ soyasaponin V (Table 1).

After soyasaponin I was hydrolyzed with recombinant Sda1 and Sde1, the degradation products were investigated by TLC using *n*-butanol: acetic acid: water (4:3:1) as the developing solvent. Only products with the same R_f value of 0.19 for the recombinant Sdn1 enzyme reaction product were detected with Sda1 and Sde1 (data not shown). These results suggest that recombinant Sda1 and Sde1 hydrolyzed soyasaponin I into soyasapogenol B and triose.

Table 1. Substrate Specificities of Saponin Hydrolases

Substrate	Specific activity (units/min/ μ g)		
	Sdn1*	Sda1	Sde1
Soyasaponin I	16.3	1.1	3.4
Soyasaponin II	5.05	0.8	2.8
Soyasaponin V	0.0057	0.0015	0.0041
Glycyrrhizin	0.371	0.2	0.018
Glycyrrhetic acid 3- <i>O</i> -glucuronide	0.088	0.25	0.076
4MU-glucuronide	nd	nd	nd

*, referring to 15.

nd, not detected.

Discussion

We clarified the characteristics and primary structures of two saponin hydrolases derived from saprophytes. The reaction velocities of both enzymes were soyasaponin I > soyasaponin II $\gg$ soyasaponin V. Since the soyasaponins consisted of the same aglycone soyasapogenol B,¹⁾ the differences in hydrolyzing velocity presumably depended on the differences in its carbohydrate moiety (Fig. 1). Therefore, these enzymes were considered to recognize carbohydrate moieties. These saponin hydrolases also hydrolyzed glycyrrhetic acid 3-*O*-glucuronide, but not 4MU-glucuronide. These results indicate that not only carbohydrate chains, but also aglycones participated in the hydrolyzing reaction.

Both Sda1 and Sde1 produced soyasapogenol B and triose from soyasaponin I, suggesting that these two enzymes have the same mode of action as previously reported for Sdn1. Furthermore, the amino acid sequences of Sda1 and Sde1 showed no similarity with any other glycoside hydrolases, an apparent homology with that of Sdn1.¹⁵⁾ These results suggest that Sda1 and Sde1 can be classified into the same family as Sdn1, and that the genes encoding them are ortholog.

Soybean saponin hydrolase was purified from *A. oryzae* KO-2.¹⁶⁾ It was found that this enzyme was a heterotetrameric protein consisting of 35 and 45 kDa, and that the ratio of substrate specificities for soyasaponin I and glycyrrhizin was 1:1.5. Glycyrrhizic acid hydrolase was purified from *A. niger* GRM3, and the molecular mass was estimated to be 150 kDa by gel filtration.²²⁾ Quite different molecular masses and substrate specificities between these glycoside hydrolases and saponin hydrolases have been clarified in series of studies by us. However, not all glycoside hydrolases hydrolyzed a typical substrate of glucuronidase, *p*-nitrophenyl or 4MU glucuronides, but all did hydrolyze saponin to aglycone and a saccharide chain without any other branch cutting.^{23,24)}

Although saponin hydrolases derived from saprophytes are considered to belong to the same family as Sdn1, Sdn1 showed apparent differences in specific activity and substrate specificity. The specific activity of Sda1 and Sde1 was only 7% and 15% respectively of that of Sdn1. Furthermore, Sda1 and Sde1 hydrolyzed

soyasaponins I and II to nearly the same extent, while soyasaponin I was more hydrolysable than soyasaponin II by Sdn1. These differences suggest that these enzymes have evolved from the same origin and are adapted to the environment of microorganisms.

Antimicrobial saponins, such as α -tomatine, avenacin A-1, and avenacosides, cause toxic effects by loss of membrane integrity.^{25,26)} Since the attached positions of carbohydrate moieties to aglycones are identical in all saponins, it is suggested that soyasaponins also have the same activity. Against them, phytopathogenic fungi such as *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *lycopersici*, *Gaeumannomyces graminis* var. *avenae*, *Septoria lycopersici*, *Stagonospora avenae*, and *V. albo-atrum* can detoxify antifungal saponins by hydrolyzing the carbohydrate moieties.^{27–37)} Rapid degradation of saponins by phytopathogenic fungi leads to rapid establishment of infection. In this case, subsequent expression of the induced defense response of host plants can be avoided or suppressed. On the other side, filamentous fungi that cannot hydrolyze saponins positively regulate the induced defense responses in the primary stage of invasion, so that infection is not established. This hypothesis is supported by reports that degradation products of saponin hydrolysis suppresses induced defense responses.^{31,38,39)}

Some strains of *N. vasinfecta* var. *vasinfecta* can infect soybean,⁴⁰⁾ and it is thought that Sdn1, which is required to detoxify saponins rapidly to establish infection, has evolved to a higher reaction speed against soyasaponins. Approximately 80% of soybean soyasaponins consist of soyasaponin I, including 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP) conjugated soyasaponins.^{41,42)} Although it is unclear whether these enzymes can degrade DDMP saponin, it is also thought that the saponin hydrolases in phytopathogens have evolved to hydrolyze the major soyasaponins rapidly. On the other hand, saprophytes obtain nutrition from harvested beans that also need to detoxify soyasaponins, but have no induced defense responses expressed in plant cells. Therefore, it is thought that this is more important for saponin hydrolase in saprophytes substrate specificities than detoxification speed.

When Sdn1 was clarified in *N. vasinfecta* var. *vasinfecta* PF1225 in a previous study, saponin hydrolases were considered to be a phytopathogen specific enzyme,¹⁵⁾ but the present study found that saprophytes have the same family of saponin hydrolases from phytopathogenic fungus. This kind of enzyme appears to be widely distributed in filamentous fungi that grow in environments requiring the degradation of saponins, irrespective of the effect on plants.

References

- 1) Yoshiki, Y., Kudou, S., and Okubo, K., Relationship between chemical structures and biological activities of triterpenoid saponin from soybean. *Biosci. Biotechnol. Biochem.*, **62**, 2291–2299 (1998).
- 2) Kinjo, J., Hatakeyama, M., Udayama, M., Tsutanaga, Y., Yamashita, M., Nohara, T., Yoshiki, Y., and Okubo, K., HPLC profile analysis of oleanene-glucuronides in several edible beans. *Biosci. Biotechnol. Biochem.*, **62**, 429–433 (1998).
- 3) Bialy, Z., Jurzysta, M., Oleszek, W., Piacente, S., and Pizza, C., Saponins in alfalfa (*Medicago sativa* L.) root and their structural elucidation. *J. Agric. Food Chem.*, **47**, 3185–3192 (1999).
- 4) Kinjo, J., Hirakawa, T., Tsuchihashi, R., Nagao, T., Okawa, M., Nohara, T., and Okabe, H., Hepatoprotective constituents in plants 14. Effects of soyasapogenol B, sophoradiol, and their glucuronides on the cytotoxicity of *tert*-butyl hydroperoxide to HepG2 cells. *Biol. Pharm. Bull.*, **26**, 1357–1360 (2003).
- 5) Ikeda, T., Udayama, M., Okawa, M., Arao, T., Kinjo, J., and Nohara, T., Partial hydrolysis of soyasaponin I and hepatoprotective effects of the hydrolytic products: study of the structure-hepatoprotective relationship of soyasapogenol B analogs. *Chem. Pharm. Bull.*, **46**, 359–361 (1998).
- 6) Miura, N., Matsumoto, Y., Miyairi, S., Nishiyama, S., and Naganuma, A., Protective effects of triterpene compounds against the cytotoxicity of cadmium in HepG2 cells. *Mol. Pharmacol.*, **56**, 1324–1328 (1999).
- 7) Sasaki, K., Minowa, N., Kuzuhara, H., Nishiyama, S., and Omoto, J., Synthesis and hepatoprotective effects of soyasapogenol B derivatives. *Bioorg. Med. Chem. Lett.*, **7**, 85–88 (1997).
- 8) Hu, J., Reddy, M. B., Hendrich, S., and Murphy, P. A., Soyasaponin I and sapogenol B have limited absorption by Caco-2 intestinal cells and limited bioavailability in women. *J. Nutr.*, **134**, 1867–1873 (2004).
- 9) Gurfinkel, D. M., and Rao, A. V., Soyasaponins: the relationship between chemical structure and colon anticarcinogenic activity. *Nutr. Cancer*, **47**, 24–33 (2003).
- 10) Rowlands, J. C., Berhow, M. A., and Badger, T. M., Estrogenic and antiproliferative properties of soy saponins in human breast cancer cells *in vitro*. *Food Chem. Toxicol.*, **40**, 1767–1774 (2002).
- 11) Berhow, M. A., Wagner, E. D., Vaughn, S. F., and Plewa, M. J., Characterization and antimutagenic activity of soybean saponin. *Mutat. Res.*, **448**, 11–22 (2000).
- 12) Kinjo, J., Yokomizo, K., Hirakawa, T., Shii, Y., Nohara, T., and Uyeda, M., Anti-herpes virus activity of Fabaceous triterpenoidal saponins. *Biol. Pharm. Bull.*, **23**, 887–889 (2000).
- 13) Ahn, K. S., Kim, J. H., Oh, S. R., Min, B. S., Kinjo, J., and Lee, H. K., Effects of oleanane-type triterpenoids from Fabaceous plants on the expression of ICAM-1. *Biol. Pharm. Bull.*, **25**, 1105–1107 (2002).
- 14) Yaguchi, T., Tsujiuchi, G., Kushida, N., and Tani, M., U.S. Patent 6878535 (Apr. 12, 2005).
- 15) Watanabe, M., Sumida, N., Yanai, K., and Murakami, T., A novel saponin hydrolase from *Neocosmospora vasinfecta* var. *vasinfecta*. *Appl. Environ. Microbiol.*, **70**, 865–872 (2004).
- 16) Kudou, S., Tsuizaki, I., Uchida, T., and Okubo, K., Purification and some properties of soybean saponin hydrolase from *Aspergillus oryzae* KO-2. *Agric. Biol. Chem.*, **55**, 31–36 (1991).

- 17) Neumann, M. J., and Dobinson, K. F., Sequence tag analysis of gene expression during pathogenic growth and microsclerotia development in the vascular wilt pathogen *Verticillium dahliae*. *Fungal Genet. Biol.*, **38**, 54–62 (2003).
- 18) Kudou, S., Tsuizaki, I., Shimoyamada, M., Uchida, T., and Okubo, K., Screening for microorganisms producing soybean saponin hydrolase. *Agric. Biol. Chem.*, **54**, 3035–3037 (1990).
- 19) Watanabe, M., Moriya, T., Aoyagi, K., Sumida, N., and Murakami, M., U.S. Patent 6277596 (Aug. 21, 2001).
- 20) Rosenfeld, J., Capdevielle, J., Guillemot, J. C., and Ferrara, P., In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal. Biochem.*, **203**, 173–179 (1992).
- 21) Yu, J., Whitelaw, C. A., Nierman, W. C., Bhatnagar, D., and Cleveland, T. E., *Aspergillus flavus* expressed sequence tags for identification of genes with putative roles in aflatoxin contamination of crops. *FEMS Microbiol. Lett.*, **237**, 333–340 (2004).
- 22) Muro, T., Kuramoto, T., Imoto, K., and Okada, S., Purification and some properties of glycyrrhizic acid hydrolase from *Aspergillus niger* GRM3. *Agric. Biol. Chem.*, **50**, 687–692 (1986).
- 23) Sasaki, Y., Morita, T., Kuramoto, T., Mizutani, K., Ikeda, R., and Tanaka, O., Substrate specificity of glycyrrhizic acid hydrolase. *Agric. Biol. Chem.*, **52**, 207–210 (1988).
- 24) Kanaoka, M., Yano, S., Kato, H., and Nakada, T., Synthesis and separation of 18 β -glycyrrhetyl monoglucuronide from serum of a patient with glycyrrhizin-induced pseudo-aldosteronism. *Chem. Pharm. Bull.*, **34**, 4978–4983 (1986).
- 25) Morrissey, J. P., and Osbourn, A. E., Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.*, **63**, 708–724 (1999).
- 26) Keukens, E. A. J., de Vrije, T., van den Boom, C., de Waard, P., Plasman, H. H., Thiel, F., Chupin, V., Jongen, W. M. F., and de Kruijff, B., Molecular basis of glycoalkaloid induced membrane disruption. *Biochem. Biophys. Acta*, **1240**, 216–228 (1995).
- 27) Quidde, T., Buttner, P., and Tudzynski, P., Evidence for different specific saponin-detoxifying activities in *Botrytis cinerea* and cloning and functional analysis of a gene coding a putative avenacinase. *Eur. J. Plant Path.*, **105**, 273–283 (1999).
- 28) Verhoeff, K., and Liem, J. I., Toxicity of tomatine to *Botrytis cinerea*, in relation to latency. *Phytopath. Z.*, **82**, 333–338 (1975).
- 29) Roldan-Arjona, T., Perez-Espinosa, A., and Ruiz-Rubio, M., Tomatinase from *Fusarium oxysporum* f. sp. *lycopersici* defines a new class of saponinase. *Mol. Plant-Microbe Interact.*, **12**, 852–861 (1999).
- 30) Lairini, K., Perez-Espinosa, A., Pineda, M., and Ruiz-Rubio, M., Purification and characterization of tomatinase from *Fusarium oxysporum* f. sp. *lycopersici*. *Appl. Environ. Microbiol.*, **62**, 1604–1609 (1996).
- 31) Ford, J. E., McCance, D. J., and Drysdale, R. B., The detoxification of α -tomatine by *Fusarium oxysporum* f. sp. *lycopersici*. *Phytochemistry*, **16**, 545–546 (1977).
- 32) Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M. J., and Osbourn, A. E., Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. *Science*, **267**, 371–374 (1995).
- 33) Sandrock, R. W., DellaPenna, D., and VanEtten, H. D., Purification and characterization of β 2-tomatinase, an enzyme involved in the degradation of α -tomatine and isolation of the gene encoding β 2-tomatinase from *Septoria lycopersici*. *Mol. Plant-Microbe Interact.*, **8**, 960–970 (1995).
- 34) Osbourn, A., Bowyer, P., Lunness, P., Clarke, B., and Daniels, M., Fungal pathogens of oat roots and tomato leaves employ closely related enzymes to detoxify different host plant saponins. *Mol. Plant-Microbe Interact.*, **8**, 971–978 (1995).
- 35) Arneson, P. A., and Durbin, R. D., Hydrolysis of tomatine by *Septoria lycopersici*: a detoxification mechanism. *Phytopathology*, **57**, 1358–1360 (1967).
- 36) Morrissey, J. P., Wubben, J. P., and Osbourn, A. E., *Stagonospora avenae* secretes multiple enzymes that hydrolyze oat leaf saponins. *Mol. Plant-Microbe Interact.*, **13**, 1041–1052 (2000).
- 37) Pegg, G. F., and Woodward, S., Synthesis and metabolism of α -tomatine in tomato isolines in relation to resistance to *Verticillium albo-atrum*. *Physiol. Mol. Plant Path.*, **28**, 187–201 (1986).
- 38) Ito, S., Eto, T., Tanaka, S., Yamauchi, N., Takahara, H., and Ikeda, T., Tomatidine and lycotetraose, hydrolysis products of α -tomatine by *Fusarium oxysporum* tomatinase, suppress induced defense responses in tomato cells. *FEBS Lett.*, **571**, 31–34 (2004).
- 39) Bouarab, K., Melton, R., Peart, J., Baulcombe, D., and Osbourn, A., A saponin-detoxifying enzyme mediates suppression of plant defences. *Nature*, **418**, 889–892 (2002).
- 40) Udagawa, S., *Neocosmospora* in Japan. *Trans. Mycol. Soc. Jpn.*, **4**, 121–125 (1963).
- 41) Hu, J., Lee, S. O., Hendrich, S., and Murphy, P. A., Quantification of the group B soyasaponins by high-performance liquid chromatography. *J. Agric. Food Chem.*, **50**, 2587–2594 (2002).
- 42) Tsukamoto, C., Shimada, S., Igita, K., Kudou, S., Kokubun, M., Okubo, K., and Kitamura, K., Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. *J. Agric. Food Chem.*, **43**, 1184–1192 (1995).