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Purification and Some Properties of Soybean Saponin Hydrolase from *Aspergillus oryzae* KO-2

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We had investigated the enzymatic hydrolysis of soybean saponins and selected soybean saponin hydrolase from *Aspergillus oryzae* KO-2. We attempted purification of this enzyme for further characterization. This enzyme was purified 1500-fold using ammonium sulfate fractionation and Sephadex G-200 gel filtrations. The enzyme was electrophoretically homogeneous and a glycoprotein by PAS staining. By gel filtration, the molecular weight of enzyme was 158,000 and SDS-PAGE showed the enzyme to have a tetrameric structure composed of heterogeneous subunits of 35,000 and 45,000. The enzyme activity was stable at temperatures below 40°C and stable from pH 5.0 to 8.0. The optimum pH was pH 4.5 to 5.0 and the optimum temperature was 50°C. The *Km* and V_{max} for soyasaponin I were 0.48 mM and 9.8 μmol/hr mg protein, respectively. After hydrolysis with the enzyme, soyasapogenol B and α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl (1→2)-D-glucuronopyranoside were released from soyasaponin I.

Soybean saponins possess some physiological activities and also have undesirable bitter and astringent tastes.[1] They have a common structure with glucuronic acid linked to the C-3 site of soyasapogenol A or B as aglycones. In our previous paper,[2] we screened microorganisms for soybean saponin hydrolase activity and selected *Aspergillus oryzae* KO-2 as having the highest enzyme activity.

Endogenous β-glucuronidases acting on acidic polysaccharides such as heparin,[3] chondroitin sulfate,[4] and hyaluronic acid[5] have been reported. They have been exploited in structure analyses of acidic polysaccharides with glucuronide bonds. Also hydrolysis of saponins by soil bacteria,[6] commercial naringenase,[7] and *Aspergillus niger*[8] have been reported, but enzymatic hydrolysis of soybean saponin has not been reported.

In our previous paper,[2] we reported that soybean saponin hydrolase was an endo-glucuronidase because no monosaccharide was detected after hydrolysis of soybean saponins by this enzyme. We therefore attempted the purification and isolation of the enzyme for further characterization. In this paper, we describe this purification and discuss some properties of the enzyme.

Materials and Methods

Materials. Crude soybean saponin fraction (11U) and soyasaponin I were prepared by the method of Shiraiwa *et al.*[9] Reduced soyasaponin I was prepared as in our previous paper,[2] Soyasaponin III and soyasapogenol B 3-*O*-β-D-glucuronopyranoside (B-G) were isolated from the hydrolysates by HPLC after soyasaponin I was partially hydrolyzed at the sugar moiety by 0.1 N HCl at 80°C for 5 days. The structures of soyasaponin I, soyasaponin III, B-G, and reduced soyasaponin I are shown in Fig. 1.

The following chemicals were purchased from commercial sources: Sephadex G-200 (Pharmacia Fine Chemicals); a calibration protein kit (catalase, aldolase, bovine serum albumin, egg albumin) (Seikagaku Kogyo); SDS-PAGE calibration protein kit (bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin) (Pharmacia Fine Chemicals); heparin, chondroitin sulfate A, and hyaluronic acid (Seikagaku Kogyo). Other chemicals were of analytical grade.

Cultivation. Aspergillus oryzae KO-2 was cultivated

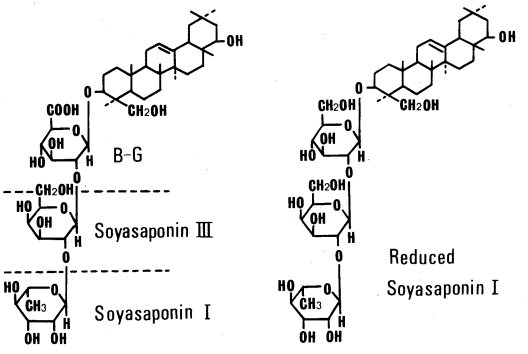

Fig. 1. Structures of Soyasaponin I, Soyasaponin III, B-G, and Reduced Soyasaponin I.

with a jar fermentor (EYELA; MBF 250M) in a medium containing 1% 11U, 4% malt extract, 2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.03% $MgSO_4 \cdot 7H_2O$, and 0.03% $CaCl_2$ at 30°C for 48 hr (pH 5.0~5.5).

Assay of activity of soybean saponin hydrolase. A half milliliter of enzyme solution was incubated with 0.5 ml of 1% 11U in 0.2 M acetate buffer (pH 5.0) at 40°C. After incubation, 1 ml of *n*-butanol containing 100 μg of glycyrrhetic acid as an internal standard was added to the reaction solution, vigorously stirred, and centrifuged. The soyasapogenol B in the *n*-butanol layer was measured by HPLC. One unit of the enzyme was defined as the amount of enzyme which produced 1 μmol of soyasapogenol B per min from soyasaponin I.

For study of the substrate specificity of enzyme, the enzyme activity was measured by the following method: after 0.1 ml of enzyme solution was incubated with 0.1 ml of 1% substrate in 0.2 M acetate buffer (pH 5.0) containing 5% Tween 80 at 40°C, the reaction mixture was heated for 5 min at 100°C. The released reducing power was measured as D-GlcUA by the method of Park and Johnson.[10]

Measurement of protein. Protein was measured by the method of Lowry *et al.*[11] with bovine serum albumin as a standard. Also chromatographic measurement of protein was done by monitoring the absorbance at 280 nm.

Polyacrylamide gel electrophoresis. Disc electrophoresis was done by the method of Davis[12] in a column of 7.5% polyacrylamide gel with a pH 9.4 buffer system. The gel was stained with Coomassie Brilliant Blue R250 for protein and PAS[13] for glycoprotein. The subunit molecular weight of the purified enzyme was examined on sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5%) by the method of Laemmli.[14]

Thin layer chromatography. Thin layer chromatography was done with a Kieselgel 60 F_{254} plate (Merck) using chloroform–methanol–water (65:35:10, v/v low layer). The components on TLC plate were colored by heating at 120°C for 10 min after spraying with 10% H_2SO_4.

High performance liquid chromatography. The quantitative analysis was done on LiChrosorb RP-18 column (5 μm 250 × 4.6 mm; Merck) using methanol–water–acetic acid (89.9:10.0:0.1, v/v) for the aglycone of soybean saponin. The flow rate was 0.5 ml/min. The instrument used was an EYELA PLC-20 with UV (205 nm) detector.

Carbon-13 nuclear magnetic resonance (^{13}C-NMR). The ^{13}C-NMR spectrum was recorded on a JEOL JMA-5600 spectrometer (100 MHz) in D_2O.

Results and Discussion

Purification of soybean saponin hydrolase

Aspergillus oryzae KO-2 was cultivated as described under Materials and Methods. The culture broth was centrifuged to remove the mycelia and the resulting supernatant was lyophilized. The lyophilized material was used as the starting crude enzyme.

The lyophilized crude enzyme (120 g) was dissolved in 500 ml of 0.02 M acetate buffer (pH 5.0) containing 0.1 M NaCl. After being stirred for 1 hr at 4°C, the solution was centrifuged to remove insoluble materials. The supernatant was used as a crude enzyme solution.

Solid ammonium sulfate was added to the crude enzyme solution (490 ml) to 20% saturation. After standing for 1 hr, the enzyme solution was centrifuged and the supernatant was adjusted to 80% saturation by further addition of solid ammonium sulfate. After this was left overnight, the resulting precipitate was collected by centrifugation and dissolved in the minimum volume of 0.02 M acetate buffer (pH 5.0) containing 0.1 M NaCl. The solution was dialyzed against the same buffer and concentrated to an appropriate volume using an ultra filter membrane (UP-20 Toyo).

The concentrated enzyme solution was put onto a Sephadex G-200 column (5 × 97 cm) that had been equilibrated with 0.02 M acetate buffer (pH 5.0) containing 0.1 M NaCl. Elution was done with the same buffer. The elution profile is shown in Fig. 2. Gel filtration, using

Table I. PURIFICATION OF SOYBEAN SAPONIN HYDROLASE FROM *Aspergillus oryzae* KO-2

	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Recovery (%)	Purification
Crude extract	11,221	941	0.084	100	1
Amm. sulfate (20–80%)	745	774	1.04	82	12
Sephadex G-200 (1st)	45.4	880	19.4	94	231
Sephadex G-200 (2nd)	2.2	279	125.3	30	1492

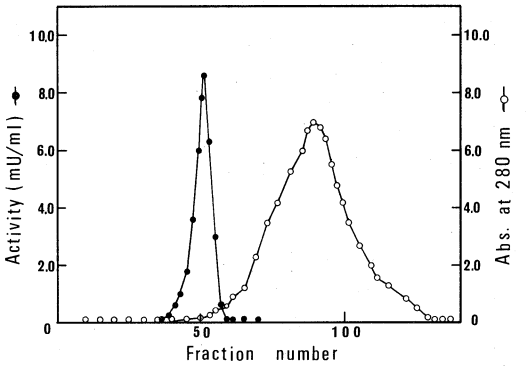

Fig. 2. Elution Profile of Soybean Saponin Hydrolase on Sephadex G-200 Gel Filtration.

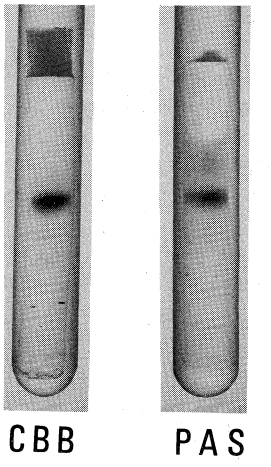

Fig. 3. Polyacrylamide Gel Electrophoresis of Purified Soybean Saponin Hydrolase.

The purified enzyme (50 μg) was electrophoresed on a column of 7.5% polyacrylamide gel with a pH 9.4 buffer system by the method of Davis. CBB, stained with Coomassie Brilliant Blue R250; PAS, stained for glycoprotein with the PAS reaction.

Sephadex G-200, led to effective separation. The active fractions were combined and concentrated using the UF membrane up to an appropriate volume.

The concentrated solution was further purified by gel filtration on a Sephadex G-200 column (2 × 83 cm) that had been equilibrated with 0.02 M acetate buffer (pH 5.0) containing 0.1 M NaCl. Elution was done with the same buffer. The active fractions were combined and used as the purified enzyme preparation. This preparation was purified about 1500-fold from the crude extract. The results of purification procedures are summarized in Table I. The increase in total activities after the 1st gel filtration was probably a result of the removal of inhibitory material.

Homogeneity of the purified enzyme preparation

The purity of the enzyme was confirmed by polyacrylamide gel electrophoresis. As shown in Fig. 3, the enzyme preparation showed a single protein band and was considered to be homogeneous. Also it was considered to be a glycoprotein as result of PAS staining.

Effects of pH and temperature on the stability and activity

The enzyme was stable from pH 5.0 to 8.0, when stored at 25°C for 15 hr (Fig. 4A). After the enzyme was incubated at various temperatures for 30 min, the residual activities were assayed at 40°C. The enzyme was stable at temperatures below 40°C and completely lost its activity at 60°C (Fig. 4B). The enzyme

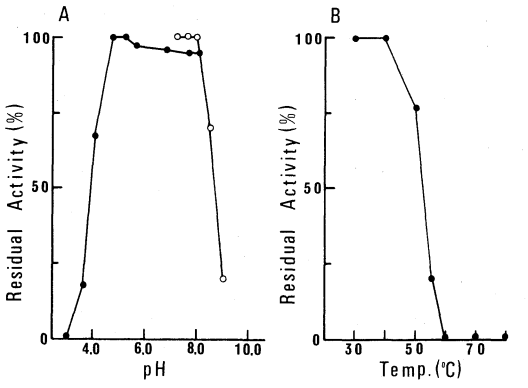

Fig. 4. Effects of pH and Temperature on the Stability of Soybean Saponin Hydrolase.

A: The enzyme was kept at 25°C for 15 hr at various pHs, and then the residual activity measured by the standard assay method. ●, McIlvaine buffer; ○, Tris–HCl buffer.
B: The enzyme in 0.02 M acetate buffer (pH 5.0) was kept at various temperatures for 30 min and then the residual activity measured at 40°C by the standard assay method.

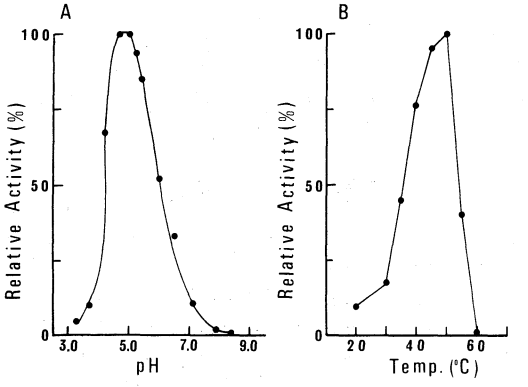

Fig. 5. Effects of pH and Temperature on the Activity of Soybean Saponin Hydrolase.

A: The enzyme activity was measured at various pHs (McIlvaine buffer) for 2 hr.
B: The enzyme activity was measured at various temperatures for 1 hr in 0.2 M acetate buffer (pH 5.0).

was most active over the range of pH 4.5 to 5.0 (Fig. 5A). The optimum temperature for enzyme activity was 50°C (Fig. 5B).

Estimation of molecular weight

The molecular weight of the soybean saponin hydrolase was estimated by gel filtration on Sephadex G-200 (2 × 83 cm) equilibrated with 0.02 M acetate buffer (pH 5.0) containing 0.1 M

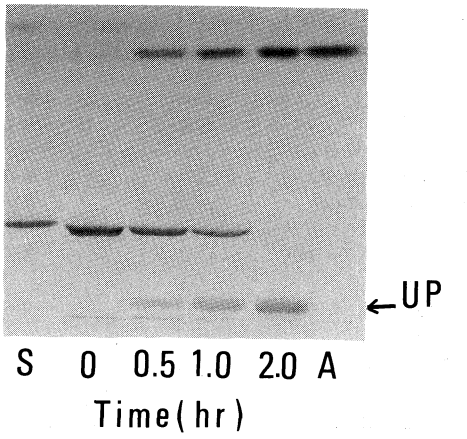

Fig. 6. Action of Saponin Hydrolase on Soyasaponin I.

The enzyme solution (0.05 U, 0.5 ml) and soyasaponin I solution (0.5%, 0.5 ml) were incubated at 40°C. After appropriate times, 200 μl of the reaction mixture was taken out and 200 μl of methanol added in to stop the reaction. S, soyasaponin I; A, soyasapogenol B.

NaCl. The molecular weight of the enzyme was estimated to be 158,000 by gel filtration using catalase, aldolase, bovine serum albumin, and egg albumin as standard proteins. After SDS-PAGE analysis using albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin as standards, protein bands of 45,000 and 35,000 were detected. We thought that this enzyme had a tetrameric structure composed of the heterogeneous subunits of 45,000 and 35,000.

Action of soybean saponin hydrolase

The action of soybean saponin hydrolase on soyasaponin I is shown in Fig. 6. After soyasaponin I was hydrolyzed by soybean saponin hydrolase, soyasapogenol B and an unknown product (UP) were released. To examine the structure of the UP, the mixture after reaction was put onto a column of Duolite S-861 resins (1 × 32 cm) and eluted with distilled water. The eluted solution was lyophilized. The lyophilized materials were dissolved in 1 ml of distilled water and further purified by Bio-gel P-2 gel filtration (1.6 × 87 cm). The structure of the purified component was analyzed by ^{13}C-NMR. The ^{13}C-NMR spectrum of the UP showed anomeric carbon sig-

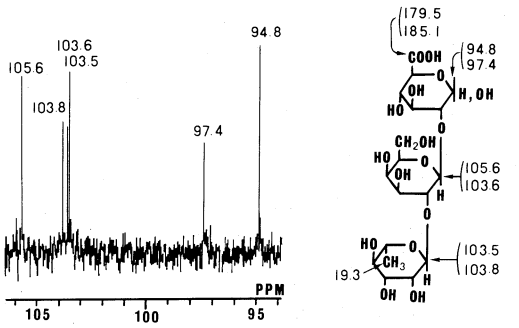

Fig. 7. ^{13}C-NMR Spectrum of UP Produced by Soybean Saponin Hydrolase.

Table II. Substrate Specificity of the Enzyme

Substrate	Relative activity (%)
Soyasaponin I	100
Soyasaponin III	71
B-G	14
Glycyrrhizic acid	150
Reduced soyasaponin I	0
Chondroitin sulfate A	0
Hyaluronic acid	0
Heparin	0
p-Nitrophenyl-β-D-glucuronoside	0
4′-Methylumbelliferyl-β-D-glucuronoside	0

nals (δ_c 94.8, 97.4, 103.5, 103.6, 103.8, 105.6), carboxyl carbon signals (δ_c 179.5, 185.1), and methyl carbon signal (δ_c 19.3), as shown in Fig. 7. From these results, we thought that the structure of the UP was α-L-rhamnopyranosyl (1→2)-β-D-galactopyranosyl (1→2)-D-glucuronopyranoside. Therefore it is evident that the soybean saponin hydrolase hydrolyzed soyasaponin I at the glucuronide bond, with release of a trisaccharide.

Substrate specificity

The enzyme activity toward various substrates was examined (Table II). 4′-Methylumbelliferyl-β-D-glucuronoside and *p*-nitrophenol-β-D-glucuronide, used as substrates of β-glucuronidase, were not hydrolyzed by this enzyme. Endo-β-glucuronidase for acidic polysaccharides such as chondroitin sulfate, hyaluronic acid, and heparin have been reported,[3-5] but these acidic polysaccharides were not hydrolyzed by this enzyme. While triterpenoid saponin with a glucuronide bond such as soyasaponin I, soyasaponin III and B-G were hydrolyzed, the relative activity decreased with decreases of the length of sugar chain. Also glycyrrhizic acid could be hydrolyzed by this enzyme, and the relative activity was 1.5-fold that of soyasaponin I. From these results, it was considered that this enzyme cleaved glucuronide linkages with large-size hydrophobic aglycone, because while triterpenoid saponins with glucuronide bonds could be hydrolyzed by this enzyme, other substrates could not be hydrolyzed. When reduced soyasaponin I was used as a substrate, it was not hydrolyzed by the soybean saponin hydrolase. This result demonstrated that the enzyme had a high substrate specificity for a glucuronide bond of glycosides.

The structural analyses of triterpenoid saponins are done with the aglycone moiety and the released monosaccharides after acid hydrolysis, but some artifacts are known to be produced by acid hydrolysis.[15] Therefore the specificity of this enzyme can be exploited in the structure analyses of glycosides with glucuronide bonds.

Kinetics

The *kinetic* analysis was done using soyasaponin I and glycyrrhizic acid as substrates (Fig. 8). The initial velocity of the reaction increased with increases of substrate concentration below 2.5 mM of soyasaponin I, and decreased with increases of the substrate concentration over 2.5 mM of soyasaponin I. This indicates that the enzyme is inhibited by the substrate at levels above 2.5 mM soyasaponin I. When glycyrrhizic acid was used as the substrate, the initial velocity of the reaction increased with increase of the substrate concentration according to Michaelis–Menten kinetics. Glycyrrhizic acid was hydrolyzed with release of glycyrrhetic acid and sugar. It was confirmed that the structure of the released sugar was β-D-glucuronopyranosyl (1→2)-β-D-glucuronopyranose by ^{13}C-NMR (anomeric carbon δ_c 94.3, 97.2, 105.0,

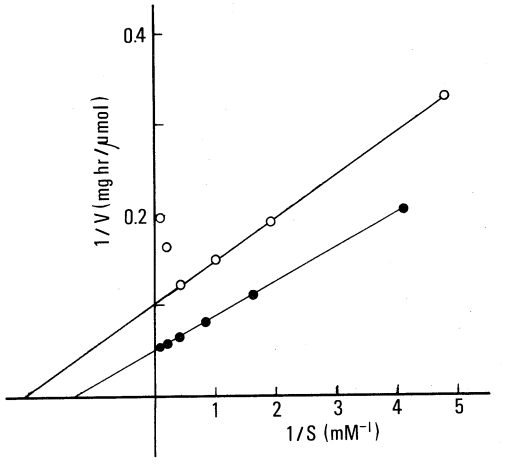

Fig. 8. Lineweaver–Burk Plots of the Reaction Velocity with Soyasaponin I and Glycyrrhizic Acid as Substrate.
●, glycyrrhizic acid; ○, soyasaponin I.

Table III. EFFECTS OF VARIOUS METAL IONS AND INHIBITORS ON SOYBEAN SAPONIN HYDROLASE

	Final concentration (mM)	Relative activity (%)
None	10	100
NaCl	10	100
KCl	10	100
$CaCl_2$	10	110
$ZnCl_2$	10	92
$MnCl_2$	10	100
$FeCl_2$	10	85
$MgCl_2$	10	102
$CuSO_4$	10	41
$AlCl_3$	10	61
$FeCl_3$	10	36
EDTA	10	110
PCMB	10	89
DFP	10	100

PCMB, *p*-chloromercuribenzoic acid; DFP, diisopropyl fluorophosphate.

106.5 carboxyl carbon δ_c 178.6, 179.5, 181.7). However D-glucuronic acid was not released by the enzyme reaction.

The V_{max} for soyasaponin I and glycrrhizic acid were 9.8 and 20.2 μmol/hr mg protein, respectively. The *Km* for soyasaponin I and glycyrrhizic acid were 0.48 and 0.74 mM, respectively, as calculated the Lineweaver–Burk plot.

Influence of various metal ions and inhibitors

The effects of various metal ions and inhibitors on the activities of the enzyme were examined (Table III). The relative activity was presented as the percentage of the control activity without metal ion or inhibitor. This enzyme was inhibited by Cu^{2+}, Al^{3+}, and Fe^{3+} and slightly inhibited by PCMB as an SH reagent, Zn^{2+}, and Fe^{2+}.

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