Preliminary Communication

A β-Rutinosidase from *Penicillium rugulosum* IFO 7242
That Is a Peculiar Flavonoid Glycosidase

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A β-rutinosidase, which was specific for releasing the disaccharide rutinose from the flavonoid glycoside rutin, was purified from *Penicillium rugulosum* IFO 7242. This enzyme had the molecular weight of 245,000, a very low optimum pH of 2.2, and the remarkable specificity that the glycosidase did not hydrolyze any other substrates like 4-nitrophenyl β-glucoside and cellobiose, but only rutin and isoquercitrin.

**Key words:** rutin; β-rutinosidase; rutinase; flavonoid glycosidase; *Penicillium rugulosum*

The flavonoids are one of the major groups in plant secondary metabolites and generally occur with sugar attachment as the glycoside.¹,² There is little knowledge of enzymes that participate in flavonoid glycoside degradation, in contrast to the biosynthetic enzymes.²-⁴ The first step of the enzymatic degradation is usually deglycosylation by flavonoid glycosidases.²,³ Most studies of their purification and properties have so far been about the glycosidases that remove monosaccharides from monoglycosides⁵-⁸ or stepwise and sequentially from glycosides containing disaccharidyl residues.³,⁹-¹¹ Although some specific glycosidases that release intact disaccharide units from flavonoid diglycosides have also been found in plants⁵,⁶,¹²-¹³ and microorganisms,¹⁴-¹⁷ they have almost never been purified and characterized, particularly from microbes.⁶,¹²,¹³,¹⁶

In order to obtain information on such specific flavonoid glycosidases, we took rutin as the substrate, which is the β-rutinoside (6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside) of the flavonol quercetin and by far the commonest flavonoid glycoside in the plant kingdom,¹¹ and then investigated microorganisms producing the glycosidases that released disaccharide rutinose from rutin. In our previous paper,¹⁸ we have reported that various glycosidases were produced in culture filtrates from rutin-degrading penicillia, and that, among these fungi, *Penicillium rugulosum* IFO 7242 especially produced a β-rutinosidase that hydrolyzed rutin to rutinose and quercetin. This communication describes the purification and several characteristics of the β-rutinosidase, which is peculiar in mode of action and the properties and is thought to be the first homogeneous preparation in microbial flavonoid glycosidases releasing the disaccharide units.

*Penicillium rugulosum* IFO 7242 mycelium was maintained on a potato dextrose agar (Difco) slant and the slant was incubated at 25°C for 7 days. The mycelium on the slant was suspended in 10 ml of sterile water and the suspension was added to a 1-liter Erlenmeyer flask containing 300 ml of the enzyme production medium. The composition of the culture medium was as before,¹⁹ including 2% rutin (Sigma) as the sole carbon source. The culture for enzyme production was done with a rotary shaker (160 rpm) at 28°C for 5 days.

β-rutinosidase activity was assayed in the reaction mixture (4 ml) containing 1 ml of 20 mM rutin in 50% (v/v) dimethyl sulfoxide (DMSO), 2 ml of 100 mM glycine-HCl (pH 2.5), and 1 ml of enzyme solution, suitably diluted. The final concentrations of rutin and DMSO were 5 mM and 12.5% (v/v) in the reaction mixture, respectively. After 5 to 20 min of incubation at 30°C, the reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid, neutralized by 1 ml of 715 mM Na₂CO₃, and added to 10 ml of methanol in order to solubilize the substrate rutin and the product quercetin. Subsequently, 1 ml of 1 mg/ml quercetin (Wako Pure Chemicals) in 40% (v/v) DMSO was added to the mixture as the internal standard substance and the final mixture was passed through a 0.2-μm membrane filter for HPLC analysis. The amount of quercetin liberated from rutin in the filtrate was measured with HPLC. One unit (U) of β-rutinosidase was defined as the amount of enzyme that produces 1 μmol of quercetin per min under these assay conditions.

The HPLC apparatus consisted of a series of LC-9A types (Shimadzu Co.) and the conditions were: the column, TSK-gel ODS-120T (I.D. 4.6 × 250 mm, Tosoh Co.); the eluting solvent, a mixture of methanol, acetic acid, and distilled water (35:10:55, v/v/v); the flow rate, 0.8 ml/min; the detective

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wavelength, 340 nm; column temperature, room temperature; injected volume, 10 µl.

Purification steps were done at 4°C. The culture filtrate of 900 ml included 914 mg protein (3,660 units), and was collected with a filter paper (No. 2, Advantec), and then was centrifuged at 10,000 g for 20 min. The supernatant was placed in cellulose tubes (separation below Mr 12,000–14,000), was concentrated to about 100 ml with polyethyleneglycol powder (PEG Mr 20,000), and was dialyzed against 20 mM acetate buffer, pH 4.0 (buffer A). The supernatant was put on a strong cation exchanger HS column (PE Applied Biosystems), which had an HPLC system and was previously equilibrated with buffer A. The column was eluted with a linear gradient of 0–500 mM NaCl in buffer A at a flow rate of 4.0 ml/min. The active fractions were pooled and concentrated to about 0.6 ml with a Centricon 30 concentrator (Amicon). The enzyme solution was continuously applied to a TSK-gel G 3000 SW column (Tosoh Co.) with an HPLC system, and equilibrated with buffer A. The active fraction was dialyzed against 20 mM acetate buffer, pH 4.8 (buffer B) and was rechromatographed on the HS column with buffer B.

Finally, 0.245 mg of the enzyme (with the specific activity of 596 U/mg) was homogeneous as judged by the criteria of native-PAGE \(^{19}\) and SDS-PAGE \(^{20}\) (Fig. 1).

The molecular weight of this enzyme was estimated to be about 245,000 by HPLC gel filtration. The subunit molecular weight was deduced to be about 65,000 from the SDS-PAGE analysis (Fig. 1). These results indicated that the purified enzyme was a homotetramer. The isoelectric point was measured to be approximately 5.0 by IEF-PAGE (Tefco Ltd.).

This \( \beta \)-rutinosidase was the most active around pH 2.2 for rutin (Fig. 2) and was stable between pH 2.0 to 11.0. The enzyme showed its highest activity at 50°C, while it was stable below 40°C.

The substrate specificity of this glycosidase was examined with TLC for detecting sugars released, and HPLC or colorimetry for measuring the activities (Table). The enzyme hydrolyzed rutin and isoorcetin, but not the others. Further, the specific activity on isoorcetin was nearly equal to that of rutin.

Several enzymes with the \( \beta \)-rutinosidase activity have been found in plants \(^{6,12-15}\) and microorganisms, \(^{14,17}\) which have individual names given by the researchers. Among those, an electrophoretically homogeneous preparation has been thus far the only rutin-degrading enzyme from tertiary buckwheat. \(^{12}\)

The tertiary buckwheat enzyme and the other two partial preparations from common buckwheat \(^{13}\) and Aspergillus flavus \(^{16}\) have molecular weights of 69,000

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**Fig. 1.** Electrophoretic Profiles of the Purified \( \beta \)-Rutinosidase. (A) Native-PAGE on the 7.5% gel. (B) SDS-PAGE on the 12% gel. Lane 1, molecular weight marker (Amersham Pharmacia Biotech), consisting of phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and \( \alpha \)-lactoalbumin (14,400); Lane 2, the purified enzyme. Both polyacrylamide gels were stained with Coomassie brilliant blue R-250.
Table. Substrate Specificity of the Purified β-Rutinosidase.

The specificity was examined by TLC, HPLC, and colorimetric analyses. TLC was done as described previously. The activities were measured with HPLC method in the test for flavonoid glycosides and colorimetric methods of 4-nitrophenol in alkaline solution or of glucose using the oxidase for the other substrates.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>Rutin (quercetin 3-O-β-rutinoside)</td>
<td>100</td>
</tr>
<tr>
<td>Isoquercitrin (quercetin 3-O-β-glucose)</td>
<td>98.7</td>
</tr>
<tr>
<td>Quercitrin (quercetin 3-O-α-rhamnoside)</td>
<td>ND</td>
</tr>
<tr>
<td>Hesperidin (hesperetin 7-O-β-rutinoside)</td>
<td>ND</td>
</tr>
<tr>
<td>Naringin (naringenin 7-O-β-neohesperidoside)</td>
<td>ND</td>
</tr>
<tr>
<td>4-Nitrophenyl α-rhamnoside</td>
<td>ND</td>
</tr>
<tr>
<td>4-Nitrophenyl β-glucose</td>
<td>ND</td>
</tr>
<tr>
<td>Methyl β-glucoside</td>
<td>ND</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>ND</td>
</tr>
</tbody>
</table>

a) β-rutinoside, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside; β-neohesperidoside, 2-O-α-L-rhamnopyranosyl-β-D-glucopyranoside.
b) Rutinoside and glucose were detected from rutin and isoquercitrin, respectively, in TLC analysis.
c) ND, not detected

to 85,000 (not reported in *A. flavus*) and their optimum pHs of 5.0 to 5.6. Hence, the β-rutinosidase of this study is distinctive with the high molecular weight and very low optimum pH, and is thought to be the first thorough preparation in microbes.

It is also noteworthy that this enzyme is specific to rutin and isoquercitrin without hydrolytic activities of 4-nitrophenyl β-glucoside and cellobiose which are generally used in the β-glucosidase assay. The activities on isoquercitrin were not reported in the above three enzymes, and 4-nitrophenyl and other phenolic β-glucosides were attacked only by the common buckwheat enzyme. This indicates that it is more necessary to evaluate not only the sugar structure but also the aglycone on the glucosidase activity.

References


