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The Activity of Rutin Degrading Enzyme from Flower Buds of Japanese Pagoda Tree

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Abstract: The rutin degrading enzyme, which was specific for releasing the disaccaride rutinose from the flavonoid glycoside rutin, was purified from the flower buds of the Japanese pagoda tree (Sophora japonica linnaeus). The crude enzyme did not act on methyl á-D-glucoside, hesperidin and cellobiose. The crude enzyme from the Japanese pagoda tree was also accompanied by low â-glucosidase activities. Based on these results, it was suggested that this enzyme is specific for rutin. This enzyme had an optimum pH value between 4.0 to 7.0, and under 50 °C, while it was stable below 5 °C.

Key words: Flavonoid, Â-glycosidase, Japanese pagoda tree, Rutin degrading enzyme

Introduction

Flavonoids are products of the secondary metabolism in plants and are of interest to the pharmaceutical and food industries because of their reported antioxidant activity (Griffiths *et al.*, 1955). Such compounds can interact with free radicals and prevent damage which radicals might otherwise cause to cell membranes and biological molecules such as DNA.

Generally, flavonoids occur with a sugar attachment as the glycoside (Harborne, 1980 and Harborne *et al.*, 1999). There is little knowledge that enzymes participate in the flavonoid glycoside degradation, in contrast to the biosynthetic enzymes (Harborne, 1980; Hosel, 1981 and Heller and Forkmann, 1994). The first step in the enzymic degradation is usually deglycosylation by the flavonoid glycosidases. Most studies of their purification and properties have so far been about the glycosidases that remove monosaccharides from monoglycosides or stepwise and sequentially from glycosides containing disaccharidyl residues. Although some specific glycosidases that release intact disaccharide units from flavonoid diglycosides have also been found in plants and microorganisms, they have almost never been purified and characterized (Hosel, 1981; Hay *et al.*, 1961; Bourbouze *et al.*, 1974 and Yasuda and Nakagawa, 1994).

In order to obtain information on such specific flavonoid glycosidases, we evaluated rutin as the substrate, which is the â-rutinoside of the flavonol quercetin. Several enzymes with the α-rutinosidase activity have been found in plants (Hosel, 1981; Yasuda and Nakagawa, 1994 and Narikawa *et al.*, 2000) Among those, an electrophoretically homogeneous preparation has been thus far the only rutin degrading enzyme from tartary buckwheat (Yasuda and Nakagawa, 1994). This paper describes the facile purification and several characteristics of the activity of an enzyme degrading rutin from the Japanese pagoda tree.

Experimental

Materials: The seeds of the Japanese pagoda tree (*Sophora japonica*) are commercially available. Rutin, quercetin, methyl â-D-glucoside, naringin and hesperidin were purchased from the Kanto Chemical Co. (Tokyo). Methyl á-D-glucoside was prepared by the reactions of D-glucose with methanol in the presence of hydrochloric acid. The purity of the product was confirmed by NMR and elemental analysis. All the solvents of guaranteed grade were purchased from the Wako Pure Chemical Co. (Osaka) and dried on molecular sieves (3Å).

Purification of Rutin Degrading Enzyme: Protein extraction was conducted by a modification of the Yasuda's method (Yasuda *et al.*, 1992). Fifty grams of dried flower buds from a Japanese pagoda tree were crushed using a homogenizer, and were extracted with 1500 ml of 0.02 M acetate buffer (pH 5) solution for 1 h, while the mixture was being stirred. The solids were centrifuged out, and the supernatant was filtered through filter paper with celite. Ammonium sulfate was added to the filtrate to 80% of saturation for 12 h at 5 °C, where some of the proteins precipitated. The precipitates were centrifuged out, and supernatant were redissolved in 100 ml of 0.02 M acetate buffer (pH 5) solution and dialyzed against deionized distilled water. Solutions of the crude protein were separately lyophilized. The obtained eight grams of protein was designated as "crude enzyme."

Assay of Rutin Degrading Enzyme Activity and Stability: The rutin degrading enzyme activity was assayed in the reaction mixture (1.2 ml) containing 7.2 mg rutin in 15% (v/v) methanol, 0.9 mg benzoic acid, 20 mM acetate buffer (pH 5), and 9 mg of enzyme. The final concentrations of rutin and benzoic acid were 8.3 mM and 6.2 mM in the reaction mixture, respectively. The mixture was incubated using a constant rotary shaking (160 rpm) at 40 °C. The initial rate of rutin degradation was measured by determination of the amount of quercetin and rutin product in the reaction mixture. Samples were taken at the intervals and the reaction was stopped by the addition of 15 μl of methanol, and the samples were analyzed using a TLC/FID analyzer (IATROSCAN new MK-5). The CHROMAROD-SIII was used as the stationary phase and chloroform/methanol/water (65/25/4 by vol.) was the mobile phase. Benzoic acid was used as the internal standard. Typical chromatograms are shown in Fig. 1. The reaction rate was calculated from the initial increase in the amount of quercetin or decrease in the amount of rutin.

Results and Discussion

Time Course of Production of Degrading Rutin: The time course of production of the degrading rutin is shown in Fig. 2. Samples withdrawn at various times from the reaction mixture containing 8.3 mM rutin and 15% (v/v) methanol were analyzed by IATROSCAN. In the first half hour of the reaction period, the rapid formation of quercetin was observed along with a sharp decrease in rutin. The rate of formation of the reaction products gradually became slower up to 2h, and at equilibrium. The concentrations of quercetin after a 24h reaction were found to be 1.3 mM.

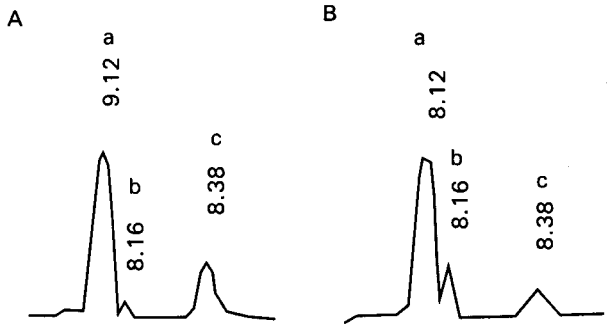

Fig. 1: Typical IATROSCAN chromatograms for the degrading of rutin to quercetin, A: after 6 minutes. B: after 30 minutes. (a: benzoic acid (internal standard); b: quercetin; c: rutin)

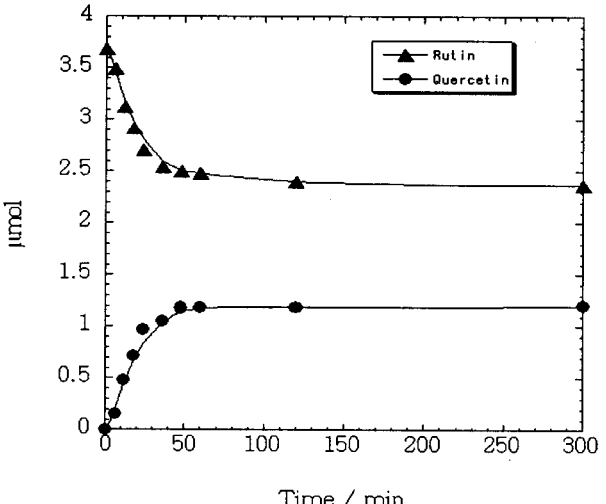

Fig. 2: Time courses of production of degrading rutin. Enzyme 9 mg, rutin 8.3 mM, 15% (v/v) methanol in acetate buffer (20 mM, pH 5.0), 40 °C. ?: rutin concentration; ?: quercetin concentration.

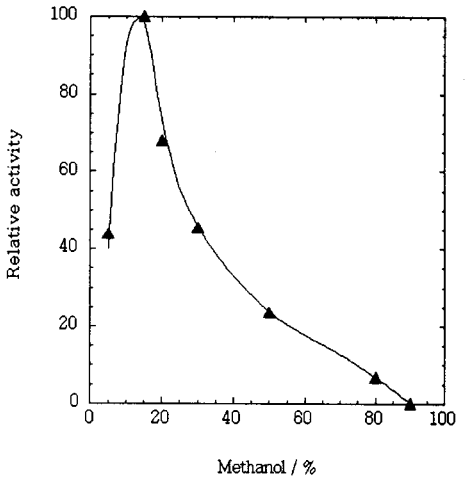

Fig. 3: Effect of methanol content on degrading of rutin. Enzyme 9 mg, rutin 8.3 mM, acetate buffer (20 mM, pH 5.0), 40 °C. Hundred percent of activity values correspond to the initial activites of 1.54 μmol•min^{-1}mg^{-1} at 15% (v/v) methanol content.

Effect of Methanol on Enzyme Activity: The effects of the methanol content on the rutin degrading reaction were investigated. As shown in Fig. 3, the reaction rate increased with the increasing methanol content, probably due to the solubility of the substrate, and caused a maximum at methanol contents around 15% (v/v). The reaction rate sharply decreased with the increasing of methanol contents above 15% (v/v). This may be the consequence of denaturation of the enzyme by methanol. As expected from this result, the rutin degrading enzyme activity was also severely retarded with the increasing methanol content. Therefore, 15% (v/v) methanol was used throughout this study.

Effect of pH Value on Enzyme Activity: After adjustment of the pH value of the reaction mixture by adding either 0.02 M NaOH or 0.02 M HCl, the enzyme assay was initiated by the addition of rutin as the

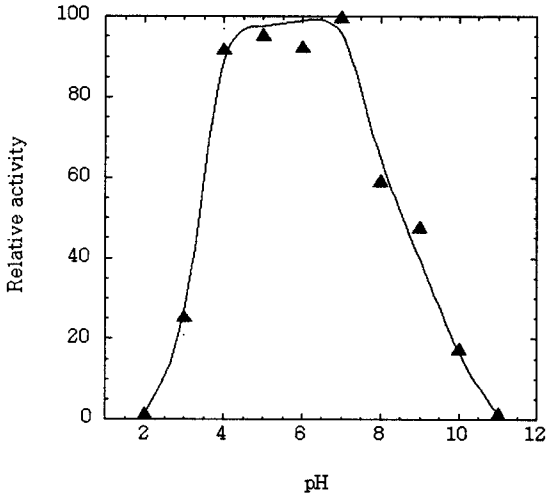

Fig. 4: pH stability of the rutin degrading enzyme. Enzyme 9 mg, rutin 8.3 mM, 15% (v/v) methanol in HCl-KCl buffer (20 mM, pH 2.0-3.0); citrate NaOH buffer (20 mM, pH 3.0-4.0); acetate buffer (20 mM, pH 4.0-6.0); phosphate buffer (20 mM, pH 6.0-9.0); glycine-NaOH buffer (20 mM, pH 9.0-11.0), 40 °C. Hundred percent of activity values correspond to the initial activites of 1.99 μmol•min^{-1}mg^{-1} at pH 7.0.

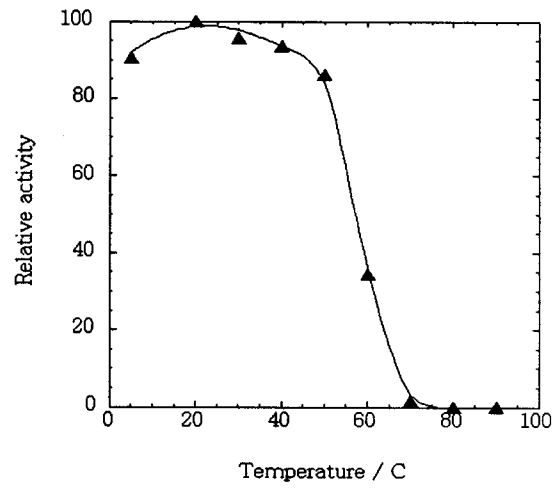

Fig. 5: Thermal stability of the rutin degrading enzyme. Enzyme 9 mg, rutin 8.3 mM, 15% (v/v) methanol in acetate buffer (20 mM, pH 5.0), 5-90 °C. Hundred percent of activity values correspond to the initial activites of 2.03 μmol•min^{-1}mg^{-1} at 20 °C.

substrate at 40 °C. The maximum enzyme activity was observed at a pH value between 4.0 to 7.0, as shown in Fig. 4.

Effect of Temperature: The enzyme activity was assayed at different temperatures at pH 5.0. As shown in Fig. 5, the optimum temperature for enzyme activity was observed under 50 °C, while it was stable below 5 °C.

Substrate Specificity: The substrate specificity of this enzyme was examined using IATROSCAN for detecting any released sugars, and measuring the activities (Table 1). The enzyme hydrolyzed rutin, methyl â-D-glucoside and naringin, but not the others. Furthermore, the specific activity on naringin was nearly equal to that of methyl â-D-glucoside. Probably due to the solubility, it was postulated that the hesperidin and the cellobiose were not hydrolyzed in the 15% (v/v) methanol-acetate buffer media. The activity of the rutin degrading enzyme from the Japanese pagoda tree has similar characteristics to that from the tartary buckwheat enzyme (Yasuda *et al.*, 1992) except for the substrate specificity. We thus conclude that these results clarified the existence of the rutin degrading enzyme contained in the Japanese pagoda tree, and this crude enzyme is specific for rutin accompanied by some α-glucosidase and naringinase activities. The present results appear of interest particularly in view of enzyme utilization. An electrophoretically homogeneous preparation is now in progress.

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