J. Microbiol. Biotechnol. (2005), **15**(3), 519–524

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Purification and Characterization of Quercitrin-Hydrolyzing α-L-Rhamnosidase from *Fusobacterium* K-60, a Human Intestinal Bacterium

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Received: August 9, 2004
Accepted: October 15, 2004

Abstract An α-L-rhamnosidase (EC 3.2.1.40.), which transforms quercitrin to quercetin, was purified from *Fusobacterium* K-60, a human intestinal anaerobic bacterium. The specific activity of the purified α-L-rhamnosidase was 2.89 mol/min/mg protein. α-L-Rhamnosidase, whose molecular size was 170 kDa by gel filtration, was composed of four subunits (M_r 41,000 Da) with pI and optimal pH values of 5.2 and 5.5–7.0, respectively. The apparent K_m and V_{max} values for p-nitrophenyl-α-L-rhamnopyranoside and quercitrin were determined to be 0.057 mM and 3.4 mol/min/mg, and 0.077 mM and 5.0 mol/min/mg, respectively. This enzyme was strongly inhibited by Cu^{2+}, Mn^{2+}, L-rhamnose, and *p*-chlormercuriphenylsulfonic acid. These findings suggest that the biochemical properties and substrate specificity of the purified enzyme are different from those of the previously purified α-L-rhamnosidase. This is the first reported purification of quercitrin-hydrolyzing α-L-rhamnosidase from intestinal bacteria.

Key words: α-L-Rhamnosidase, quercitrin, *Fusobacterium* K-60, intestinal bacteria

Most herbal medicines are orally administered, and most components of these medicines are inevitably brought into contact with intestinal microflora in the alimentary tract and are transformed by the intestinal bacteria before their absorption from the gastrointestinal tract [1, 4, 9, 14, 15]. Many researchers have suggested that the metabolism of these components should be related to their pharmacological actions. Bae *et al.* [2] reported that hypoglycemic and cytotoxic activities of oral administration of tectoridin were due to tectorigenin metabolized by intestinal microflora, and Wakabayshi *et al.* [22] reported that the antimetastatic action of ginsenosides is due to their metabolites.

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Related to the metabolism of orally administered flavonoid rhamnoglycoside of natural product, Bokkenheuser *et al.* [5] isolated *Bacteroides* spp., which produces α-rhamnosidase, β-glucosidase, and β-galactosidase, from human intestinal microflora, and reported that rutin and robinin are hydrolyzed by these bacteria. We also isolated *Bacteroides* JY-6, a human intestinal anaerobic bacterium, which produced α-L-rhamnosidase and β-D-glucosidase. *Bacteroides* JY-6 transformed flavonoid rhamnoglucosides, such as rutin, hesperidin, poncirin, and naringin, to their aglycones [12]. However, these bacteria did not hydrolyze flavonoid-α-L-rhamnosides such as quercitrin, which is a main component of *Houttuynia cordata* (Saururaceae). Therefore, we screened for quercitrin-hydrolyzing bacteria from human intestinal bacteria, and isolated a bacterium, *Fusobacterium* K-60, which transforms quercitrin to quercetin [13].

Many microbes have been studied for their potential to produce glycosidases. However, little is known about microbal α-L-rhamnosidases, particularly quercitrin-hydrolyzing enzymes. α-L-Rhamnosidase (EC 3.2.1.40.), which is able to hydrolyze L-rhamnose of α-glycosidic bond, is widely distributed in animal tissues [16], plants [6], fungi [11, 18–20], and bacteria [5, 11]. It has been purified from a number of mammalian tissues, plants, and bacteria. However, the quercitrin-hydrolyzing α-L-rhamnosidases have not yet been purified. In the present study, quercitrin-hydrolyzing α-L-rhamnosidase was purified from *Fusobacterium* K-60 and the properties of the purified α-L-rhamnosidase were compared with those of the enzymes purified previously from intestinal bacteria.

MATERIALS AND METHODS

Materials

p-Nitrophenyl-α-L-rhamnopyranoside (PNR), p-nitrophenyl-α-D-glucopyranoside, o-nitrophenyl-α-D-glucopyranoside,

p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenylacetate, p-nitrophenylphosphate, p-nitrophenylsulfate, naringin, neohesperidin, hesperidin, rutin, L-rhamnose, L-mannose, D-mannose, L-fucose, tosyl-L-lysine chloromethyl ketone (TLCK), iodoacetic acid (IAA), N-ethylmaleimide (NEM), *p*-chloromercuriphenylsulfonic acid (PCMS), carbodiimide, paraoxon, ethylenediamine tetraacetic acid (EDTA), dithiothreitol, β-mercaptoethanol, and tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma Chem. Co. (U.S.A.). High molecular weight markers for gel filtration chromatofocusing gels, polybuffer, and Sephacryl S-300 were obtained from Pharmacia Fine Chem. Co. (Sweden). The tryptic soy broth was purchased from Difco Co. (U.S.A.). All other chemicals were of analytical reagent grade.

Assay of α-L-Rhamnosidase Activity

The reaction mixture (total volume of 0.5 ml) contained 0.2 ml of 1 mM PNR, 0.2 ml of 20 mM phosphate buffer (pH 7.0), and 0.1 ml of the enzyme suspension. The reaction mixture was incubated at 37°C, then stopped by the addition of 0.2 N NaOH (0.5 ml), and absorbance at 405 nm was measured by UV spectrophotometery (Shimadzu UV-120-02, Japan) [8].

In the case of natural rhamnoglucosides as the substrate, the reaction mixture contained 0.2 ml of 1 mM substrate, 0.2 ml of 20 mM phosphate buffer (pH 7.0), and 0.1 ml of the enzyme suspension. After the reaction mixture was extracted with 0.5 ml of ethyl acetate, the ethyl acetate layer was applied to thin layer chromatography [the developing solvent of TLC (Merck, 5715) was $CHCl_3$/MeOH (4/1)] and quantified with a TLC scanner (Shimadzu model CS-9000, Japan; Wavelength, 254 nm).

One unit of the enzyme activity was defined as the amount of the enzyme required to catalyze the formation of 1.0 μmole of product (p-nitrophenol or other substrate) per minute under the standard assay conditions. Specific activity was defined in terms of units per mg protein.

Purification of α-L-Rhamnosidase from *Fusobacterium* K-60

Fusobacterium K-60, which was isolated from human intestinal microflora, was subcultured in the synthetic medium, which consisted of tryptic soy broth containing 0.1% ascorbic acid, 0.01% sodium thioglycolate, and pH adjusted to 7.2. The subcultured medium (100 ml) was inoculated in 10 l of the same medium, and then cultured at 37°C for 15 h. The cultivated medium was centrifuged at 5,000 ×*g* for 20 min at 4°C. The collected bacteria were washed with saline. The resulting precipitate was suspended in 150 ml of 20 mM phosphate buffer (pH 7.0), and then disrupted by an ultrasonicator. The sonicated suspension was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant (150 ml) was used as a crude enzyme suspension. It was then fractionated with 30% to 70% ammonium sulfate saturation and the suspension was centrifuged at 10,000 ×*g* for 30 min. The resulting precipitate was dissolved in 80 ml of 20 mM phosphate buffer (pH 7.0) containing 1.5 M KCl. All purification procedures were performed at 4°C. The enzyme suspension was applied to a column of Butyl-toyopearl (3×15 cm) previously equilibrated with 20 mM phosphate buffer (pH 7.0) containing 1.5 M KCl. The column was washed with 100 ml of the same buffer, and then a linear gradient elution was carried out with 150 ml of the same buffer and 150 ml of 20 mM phosphate buffer. Fractions (4.5 ml each) were collected, and monitored by measuring absorbance at 280 nm and α-L-rhamnosidase activity. The active fractions (Fr. Nos. 31–42) were pooled, and the pooled sample was dialyzed twice against 2 l of 50 mM sodium phosphate buffer (pH 7.0) for 12 h. The dialysate was applied to the hydroxyapatite column (2.8×3 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was eluted with 240 ml of a linear gradient formed by 50 to 250 mM sodium phosphate buffer (fraction volumes 4.8 ml). The active fractions (Fr. Nos. 66–74) were pooled and concentrated at 9 psi and 4°C, using Avantec pressure filtration equipped with PM-10 membranes to approximately 1.5 ml. The concentrate was applied to a Sephacryl S-300 HR column (2.6×70 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0), and the column was eluted (flow rate, 0.5 ml/min; fraction volumes 0.93 ml). The active fractions (Fr. Nos. 75–82) contained homogeneous α-L-rhamnosidase, as determined by native and denatured PAGE.

Protein Determination

Protein was measured by the method of Bradford's protein assay kit, using bovine serum albumin as a standard [3, 7].

Table 1. Summary of the steps for purifying α-L-rhamnosidase from *Fusobacterium* K-60.

Step	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg)
Crude extract	182.9	2,606.4	0.1
Ammonium sulfate fractionation (70%)	348.7	1,237.1	0.3
Butyl-Toyopearl column chromatography	118.6	111.7	1.1
Hydroxyapatite column chromatography	32.4	0.55	58.9
Sephacryl S-300 column chromatography	24.8	0.049	506.1
Q-Sepharose column chromatography	15.8	0.006	2,872.7

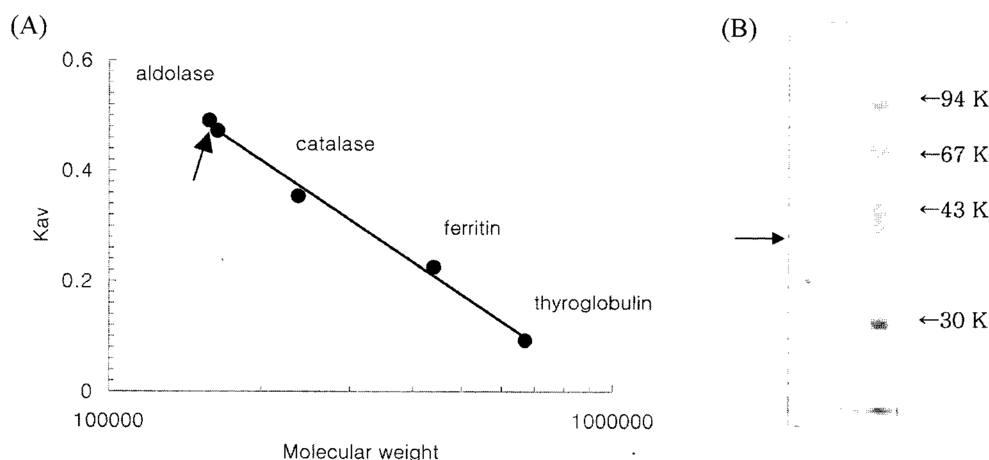

Fig. 1. Molecular weight determination by gel filtration (A) and SDS-polyacrylamide gel electrophoresis (B).
Arrow indicates the purified α-L-rhamnosidase.

Polyacrylamide Gel Electrophoresis

SDS-PAGE (10%) and non-SDS-PAGE were performed according to the modified method of Laemmli [10, 17], and the gel was stained with Coomassie blue. The enzyme activity staining was performed as follows; the electrophoresed gel was placed on filter paper, which was immersed with a reaction mixture (0.5 ml of 0.5 mM 4-methylumbelliferyl-α-L-rhamnopyranoside and 1.5 ml of 20 mM phosphate buffer, pH 7.0), and the paper was incubated at 37°C. The enzyme-active band exhibited white fluoroscence.

RESULTS

Purification of α-L-Rhamnosidase from *Fusobacterium* K-60

The α-L-rhamnosidase was purified about 30,000-fold from a crude extract of *Fusobacterium* K-60 through the procedures shown in Table 1, with a yield of 8.6%. The specific activity of the purified enzyme was 2.83 units/mg protein. A single band was detected by SDS-PAGE (Fig. 1).

General Properties

The molecular mass of the purified α-L-rhamnosidase was determined to be about 170 kDa by the Sephacryl S-300 gel filtration method, and that of the subunit was found to be 41 kDa by SDS-PAGE (Fig. 2).

The pH-activity profile indicated the optimal pH of the α-L-rhamnosidase to be 5–6.5, using PNR and quercitrin as substrates (Fig. 3). The pI of the enzyme was 5.2, as determined by chromatofocusing column chromatography. As listed in Table 2, Cu^{2+} and Mn^{2+} inhibited the enzyme activity, however, most metal ions tested did not affect the enzyme activity. Effect of 6-deoxyhexoses and hexoses on K-60 α-L-rhamnosidase

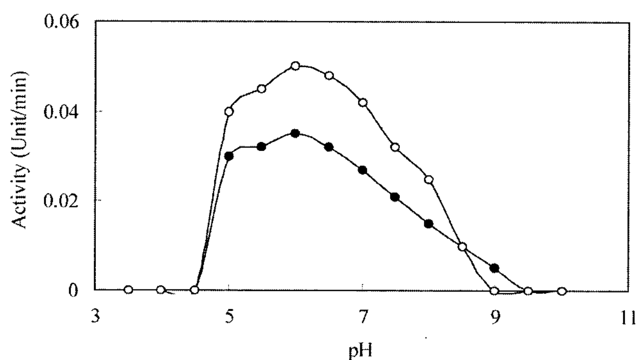

Fig. 2. pH profile of K-60 α-L-rhamnosidase activity.
Enzyme activity was assayed under the standard conditions. Substrates used were as follows: open circle, quercitrin; closed circle, p-nitrophenyl α-L-rhamnopyranoside. Buffer used: pHs 3–6, 0.1 M acetate buffer; pHs 6–7.5, 0.1 M phosphate buffer; pHs 7.5–9.0, 0.1 M Tris-HCl buffer; pHs 9–11, 0.1 M glycine-NaOH buffer.

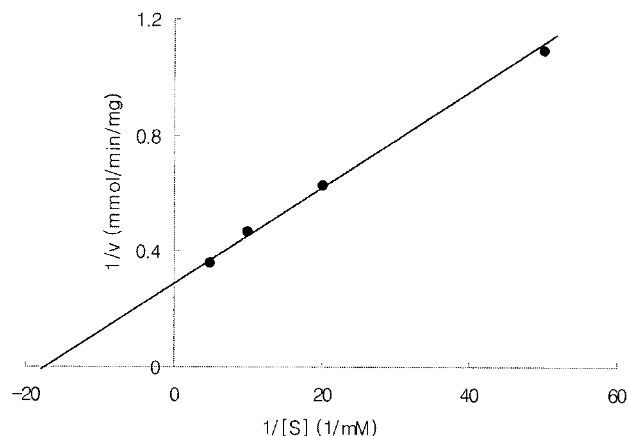

Fig. 3. Linewweaver-Burk plot of K-60 α-L-rhamnosidase for PNR.

Table 2. Effect of metal ions on Fusobacterial α-L-rhamnosidase activity.

Metal ion[a]	Residual activity (%)[b]
None	100
Zn^{2+}	68
Cu^{2+}	12
Ca^{2+}	111
Ni^{2+}	125
Mg^{2+}	118
Co^{2+}	116
Mn^{2+}	11
Pb^{2+}	120
EDTA	144

[a]Final concentration was 1 mM. Metal ions as chloride salts were used.
[b]0.05 unit of the enzyme was taken as 100%.

activity was investigated (Table 3): L-rhamnose was found to be a competitive inhibitor of the enzyme a with K_i value of 0.12 mM.

When the effect of chemical modifying agents on K-60 α-L-rhamnosidase activity was measured, the enzyme was completely inhibited by PCMS, which bonds covalently to L-cysteine (Table 4). The other reagents did not inhibit the enzyme. The optimum pH for stable storage of the purified α-L-rhamnosidase was 5.5–6.0, and the purified enzyme was unstable even at –20°C and 4°C (data not shown).

Substrate Specificity

The substrate specificity of the purified enzyme for various rhamnoglucosides is presented in Table 5. The present α-L-rhamnosidase hydrolyzed quercitrin, poncirin, naringin, rutin, and hesperidin to form quercetin, poncirenin, prunin, quercetin-3β-D-glucopyranoside, and hesperetin-7β-D-glucopyranoside, respectively. The best substrate was quercitrin, followed by PNR, hesperidin, poncirin, naringin, and rutin. However, other glycosides, such as p-nitrophenyl-α-D-glucopyranoside, o-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucuronide, and p-nitrophenyl-β-D-galactopyranoside, were not effective as substrates. The apparent K_m and V_{max} values for quercitrin were 0.077 mM and 5.0 units/mg protein, respectively, as determined by Lineweaver-Burk plots.

Table 3. Sugars that inhibit α-L-rhamnosidase purified from *Fusobacterium* K-60.

Sugar[a]	Residual activity[b] (%)	K_i (mM)
L-Rhamnose	26.3	0.124
L-Mannose	95	–[c]
D-Mannose	119	–
D-Glucose	121	–
D-Galactose	133	–
L-Fucose	87	–
Tris	13.2	0.010
Saccharic acid-1,4-lactone	15.8	0.012

[a]Final concentration was 0.2 mM.
[b]0.05 unit of the enzyme was taken as 100%.
[c]Not measured.

Table 4. IC_{50} values of chemical modifying agents on α-L-rhamnosidase purified from *Fusobacterium* K-60.

Chemical[a]	Residual activity (%)[b]
TLCK	78
IAA	101
NEM	82
PCMS	11
Carbodiimide	74
Paraoxon	89
EDTA	98
Mercaptoethanol	102

[a]Final concentration was 1 mM.
[b]0.05 unit of the enzyme was taken as 100%.

DISCUSSION

As is well known, flavonoid rhamnoglucosides are widely distributed in most fruits, vegetables, and herbal medicinal plants. They are resistant to boiling and fermentation, and more than 1 g are ingested daily by humans. Related to the metabolism of these compounds, Bokkenheuser *et al.* [5] isolated *Bacteroides* spp. from human intestinal microflora, and we also previously purified the α-L-rhamnosidase from *Bacteroides* spp. isolated from human intestinal bacteria [8]. These bacteria and their α-L-rhamnosidase potently hydrolyze flavonoid rhamnoglycosides such as naringin, poncirin, hesperidin, and rutin. The bacteria previously isolated could not convert quercitrin to quercetin; however, when incubated with human intestinal microflora, quercitrin was easily metabolized to quercetin. Based on this finding, we isolated anaerobic *Fusobacterium* K-60, the bacterium transforming quercitrin to quercetin, from human intestinal microflora [13]. The specific activity of the homogeneous α-L-rhamnosidase purified from *Fusobacterium* K-60 was 2.89 units/mg protein. The best substrate of the α-L-rhamnosidase was quercitrin, followed by PNR. The presently purified enzyme hydrolyzed quercitrin more potently than other natural rhamnoglycosdes such as hesperidin, poncirin, and naringin. Okada *et al.* [21] reported the substrate specificity of *Aspergillus niger* α-rhamnosidase T and N towards flavonoid glycosides: α-L-rhamnosidase T was highly specific on quercitrin, compared with naringin, and α-rhamnosidase N was highly specific on naringin (not quercitrin). The present enzyme seems to be similar to that of *Aspergillus niger* α-L-rhamnosidase T, but is different from the enzymes previously reported, including fungal α-L-rhamnosidase and *Bacteroides* JY-6 α-L-rhamnosidase [5, 8].

Table 5. V_{max}, K_m, and K_{cat} values of α-L-rhamnosidase purified from *Fusobacterium* K-60.

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	K_{cat} (s^{-1})
p-Nitrophenyl-α-L-rhamnopyranoside	3.4	0.057	9.64
Quercitrin	5.0	0.077	14.17
Hesperidin	0.52	0.022	1.47
Naringin	0.34	0.021	0.96
Poncirin	0.35	0.020	0.99
Rutin	0.07	0.028	0.20

The general properties of the present enzyme, such as molecular mass, pI, and optimal pH, were different from those of the α-L-rhamnosidases from *Bacteroides* JY-6 and *Aspergillus niger. Fusobacterium* K-60 α-L-rhamnosidase was strongly inhibited by Cu^{2+} and Mn^{2+}. These properties were also quite different from those of the enzymes previously purified from *Aspergillus* spp. and *Bacteroides* spp.

Fusobacterium K-60 α-L-rhamnosidase was competitively inhibited by L-rhamnose and PCMS, which forms a covalent bond with L-cysteine. This result suggests that the configuration of the methyl group in 6-position of sugar plays an important role in substrate binding to the enzyme, whose active site should contain the L-cysteine residue. These properties of *Fusobacterium* K-60 α-L-rhamnosidase are similar to those of *Bacteroides* JY-6 α-L-rhamnosidase. Finally, we suggest that the present enzyme may play an important role in the metabolism of quercitrin as well as flavonoid rhamnoglycosides, which are found in most fruits, vegetables, and herbal medicines.

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