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Purification and Some Properties of Rutinosidase from *Arthrobacter* sp.

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Arthrobacter sp.가 생산하는 Rutinosidase의 정제 및 특성

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The several glycoside hydrolysing enzymes related to rutin degradation are found to be rhamnosidase, glucosidase and rutinosidase. Rutinosidase was purified to electrophoretic homogeneity from cell extracts of rutin-degrading strain, MT-57, which was identified as a *Arthrobacter* sp. Its molecular weight was estimated to be 42,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 40,000 by gel filtration. The optimum pH for enzyme was found to be 7.5, and relatively stable in alkaline solution. The optimum temperature for enzyme was 45°C, being stable up to 50°C for 20 min. The Km value of enzyme for rutin was 0.5 μM. The enzyme activity was increased by the chelating agent such as EDTA, NaN_3, and 8-hydroxyquinoline, was strongly inhibited by Co^{2+}, Ni^{2+}, and Cu^{2+}. The enzyme had high substrate specificity in the rutinoside.

Microbial transformation of organic compounds is currently an important research pursuit, since the ability of microorganism to enzymatically transform naturally occurring organic compounds to other substances is well known. A number of these studies (1-9) using fungi and bacteria have been reported on the biotransformation of flavonoid compounds which are common constituents of higher plants. Simpson *et al.* (10) investigated the degradation of rutin by *Aspergillus flavus*. They reported that *Aspergillus flavus*, when grown on rutin, produces partial purified extracellular enzymes which degraded rutin to carbon monoxide, rutinose, phloroglucinol carboxylic acid, and protocatechuic acid. The first of the degradative reactions is the cleavage by "rutinase" of the glycosidic bond to yield rutinose and quercetin. On the other hand, there are few reports concerning the bacterial degradation of rutin (11). In this point of view, we have studied on the oxidation products derived from rutin and confirmed by the thin layer chromatography (TLC) that the several sugars such as rhamnose, glucose and rutinose, produced from rutin by the crude extracts of the rutin-degrading bacteria. This result shows that the several glycoside hydrolysing enzymes are involved in degradation of rutin.

This paper describes the enzymes catalyzing the hydrolysis of sugar moiety of rutin, the purification and some properties of the rutinosidase which are intracellular enzymes capable of degrading rutin to rutinose and quercetin.

Materials and Methods

Indentification of strain MT-57

Strain MT-57 used in this work was isolated from a soil sample by means of selected enrichment culture. The strain was identified as *Arthrobacter* sp. accord-

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ing to the "Bergey's Manual of Determinative Bacteriology 8th ed." (12).

Maintenance and growth of the organism

Stock cultures of strain MT-57 were maintained on the culture agar medium containing 2% rutin, and stored at 4°C. The basal medium had the following composition (g/l); NH_4NO_3, 4.0; KH_2PO_4, 1.5; Na_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.005; yeast extract, 0.005; and tap water to make 900 ml. The initial pH was adjusted to 7.0. The culture medium was sterilized at 120°C for 10 min, and then 100 ml of sterilized 2% (w/v) rutin solution was added.

A large scale cultivation was carried out in 5 l Erlenmeyer flasks containing 1 l of the culture medium (4% rutin) at 30°C with reciprocal shaking. The medium in 5-liter Erlenmeyer flasks was inoculated by transfer of 100 ml of shake-cultured broth from a 500 ml shaking flask. After 12 hrs the cells were harvested, washed with 50 mM potassium phosphate buffer and stored at −80°C until required.

Enzyme assay

Rutinosidase activities were measured by reducing sugar cleaved and quercetin formed from rutin. The reaction mixture (1 ml), consisting of 0.1 ml enzyme solution and 0.9 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 μmol substrate, was incubated at 30°C for 30 min. Reducing sugar cleaved from rutin after reaction was measured by Somogi-Nelson method (13, 14) at 660 nm. Quercetin formed from the substrates was measured spectrophotometrically at 450 nm after reaction was ended by the addition of 3 ml boric acid solution (0.2 M pH 9.8) (10). One unit of enzyme activity was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmol of rutin per minute.

Protein determination

During the course of enzyme purification, the protein eluted from the column was measured by the absorbance at 280 nm and the protein in the pooled sample was measured by Bio-Rad protein assay using bovine serum albumin as a standard.

Enzyme purification

Step 1. Extraction: All operation for cell extracts were performed at 0°C to 4°C. 50g (wet weight) of frozen cells were suspended in 400 ml of 50 mM potassium phosphate buffer and disrupted in 80 ml portions by treatment on ice at 2 A for 12 min with Kubota insonator (Model 200 M). The suspension centrifuged (14,000×g for 10 min) and the supernatant solution was collected. The supernatant solution was ultracentrifuged (140,000×g for 10 min) and the supernatant solution after ultracentrifugation was as the crude extract.

Step 2. Streptomycin sulfate treatment: The crude extract was treated with 5% (w/v) streptomycin sulfate solution by dropwise addition until no more precipitate formed. After being kept on ice for 1 hr, the precipitate was removed by centrifugation at 16,000×g for 10 min.

Step 3. Ammonium sulfate fractionation: The supernatant from step 2 was brought to 30% saturated solution with respect to $(NH_4)_2SO_4$ by the slow addition of powdered $(NH_4)_2SO_4$ with stirring, the pH being kept at 7.0 by the dropwise addition of 1 N NH_4OH solution. And then allowed to equilibrate at 4°C for 1 hr. The precipitate formed was removed after centrifugation at 16,000×g for 10 min. The supernatant was made to a 50% saturated solution by the more addition of powdered $(NH_4)_2SO_4$. The resulting precipitate, after equilibration as before, was collected by centrifugation at 16,000×g for 10 min and then dissolved in a minimum volume of 10 mM potassium phosphate buffer. Ammonium sulfate was removed by dialysis against the same buffer overnight.

Step 4. Chromatography on a hydroxyapatite column: The $(NH_4)_2SO_4$ fraction was applied to the top of a column (3 by 10 cm) of hydroxyapatite previously equilibrated with 10 mM potassium phosphate buffer. The proteins were eluted with the same buffer and the fractions of 15 ml were collected and each fraction was assayed for enzyme activity. The active fractions were collected and subjected to the next procedure without dialysis.

Step 5. Chromatography on a DEAE-Toyopearl 650S column: The enzyme solution was brought to 0.15 M NaCl with gently stirring, the pH being kept at 7.0 by the dropwise addition of 1 N NH_4OH solution. The enzyme solution was applied to the top of a column (2 by 22 cm) of DEAE-Toyopearl 650S previously equilibrated with 10 mM potassium phosphate buffer supplemented with 0.15 M NaCl. The column was washed thoroughly with the same buffer (supplemented with 0.15 M NaCl) until no protain was de-

tected in the eluate. The protein was eluted with a 0.15-0.45 M linear NaCl gradient in 400 ml of the same buffer. Fractions of 10 ml were collected and each fraction was assayed for enzyme activity. The enzyme was eluted with a linear gradient of 0.18-0.25 M NaCl. The active fractions were combined and dialyzed against 10 mM potassium phosphate buffer for overnight.

Step 6. Chromatography on a Butyl-Toyopearl 650M column: The dialyzed enzyme solution was made to a 20% saturated solution with respect to $(NH_4)_2SO_4$ by the addition of powdered $(NH_4)_2SO_4$ with gently stirring, the pH being kept at 7.0 by the dropwise addition of 1 N NH_4OH solution. The enzyme solution was applied to the top of a column (2 by 9.3 cm) of Butyl-Toyopearl 650 M previously equilibrated with 10 mM potassium phosphate buffer supplemented with 20% $(NH_4)_2SO_4$. The column was washed thoroughly with the same buffer (supplemented with 20% $(NH_4)_2SO_4$), until no protein was detected in the eluate. The protein was eluted with a 20%-0% linear $(NH_4)_2SO_4$ gradient in 240 ml of the same buffer. Fractions of 8 ml were collected and each fraction was assayed for enzyme activity. The enzyme was eluted with a linear gradient of 12-18% and 5-2% $(NH_4)_2SO_4$. The active fractions were combined and concentrated further to 5 ml by ultrafiltration after dialyzed against 50 mM potassium phosphate buffer for overnight.

Step 7. Chromatography on a Toyopearl HW50S column: The concentrated enzyme solution was put on a column (4.4 by 71.5 cm) of Toyopearl HW50S that had been eqilibrated with 50 mM potassium phosphate buffer (pH 7.0), and enzyme was eluted with the same buffer at flow rate of 48 ml per hr. Fractions of 5 ml were collected and each fraction was assayed for enzyme activity. The active fractions were combined and dialyzed against 10 mM potassium phosphate buffer for 6 hr.

Step 8. Chromatography on a DEAE-Cellulose DE-52 column: The dialyzed enzyme solution was applied to the top of a column (2 by 10 cm) of DEAE-Cellulose DE52 previously equilibrated with 10 mM potassium phosphate buffer. The column was washed thoroughly with the same buffer until no protein was detected in the eluate. The protein was eluted with a 0-0.5 M linear NaCl gradient in 360 ml of the same buffer. Fractions of 5 ml were collected and each fraction was assayed for enzyme activity. The enzyme was eluted a linear gradient of 0.25-0.3 M NaCl. The active fractions were combined and dialyzed against 10 mM potassium phosphate buffer for 6 hr. The dialyzed enzyme solution was stored at −80°C. A typical purification scheme is given in Table 1.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Williams (15). Electrophoresis was performed with 7% gels and barbitarl-tris buffer (pH 7.0), at a current of 2 mA/gel. Experiments were performed at room temperature and terminated when the bromo phenol blue marker reached the lower end of the gel. Protein was stained by immersing the gel in a 0.25% solution of Coomassie Brilliant Blue R 250 in methanol-acetic acid-water (5:1:5, v/v/v). Excess stain was removed by immersion in methanol-acetic acid-water (3:1:6, v/v/v).

Measurement of molecular weight

Gel filtration on Toyopearl HW50S: Gel filtration

Table 1. Purification of rutinosidase from *Arthrobacter* sp. MT-57.

Step	Total activity (m Units)	Total protein (mg)	Specific activity (m Units/mg protein)	Purification (fold)	Yield (%)
Crude extracts	75,400	2,400	31	1	100
Streptomycin treatment	68,100	2,030	34	1.1	90
$(NH_4)_2SO_4$ fractionation	29,000	157	185	5.9	39
Hydroxyapatite	5,280	24	220	7.0	7.0
DEAE-Toyopearl 650S	1,290	4.2	307	9.8	1.7
Butyl-Toyopearl 650 M	870	1.1	788	25.0	1.1
Toyopearl HW50 S	710	0.7	1,010	32.0	0.9
DEAE-Cellulose DE 52	430	0.2	2,160	69.0	0.57

was done on Toyopearl HW50S (4.4 by 71.5 cm) with 50 mM potassium phosphate buffer, pH 7.0, according to the method of Andrews (16).

SDS-PAGE: SDS-PAGE was done by the method of Laemmli (17) with 12% acrylamide containing 0.1% SDS. Samples were boiled for 2 min or incubated for 12 hr with 1% SDS and 10% glycerol in the presence or absence of 1% 2-mercaptoethanol. Bromophenol blue was used at a front marker. Protein was stained with Coomassie Brilliant Blue R250 and the gels were destained by immersion in a solution of acetic acid-methanol-water (1:3:6, v/v/v).

Results and Discussion

Bacterial strain

MT-57 was identified as a strain of *Arthrobacter* on the basis of its morphological and biological characteristics. It is gram-negative, non motile rod. The organism gave a positive reaction for catalase and negative reaction for oxidase. Indole production, H_2S production, were positive, nitrate reduction, OF test were negative. Neither acid nor gas were formed when MT-57 grew on glucose, fructose, maltose, mannose, cellobiose, sucrose, lactose, rhamnose, trehalose, salicin, and soluble starch.

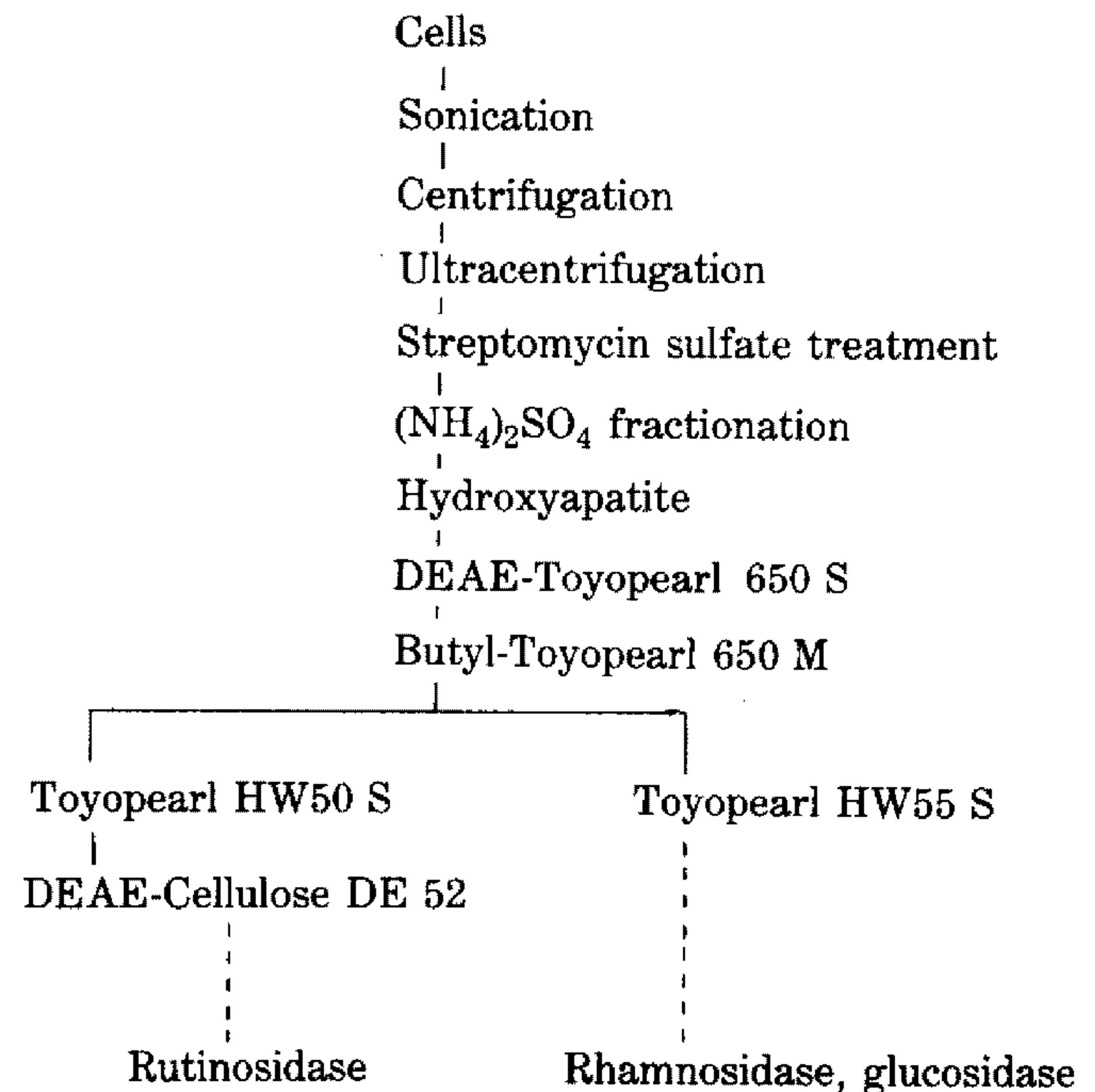


Fig. 1. Procedure for purification of glucoside hydrolysing enzymes from *Arthrobacter* sp. MT-57 .

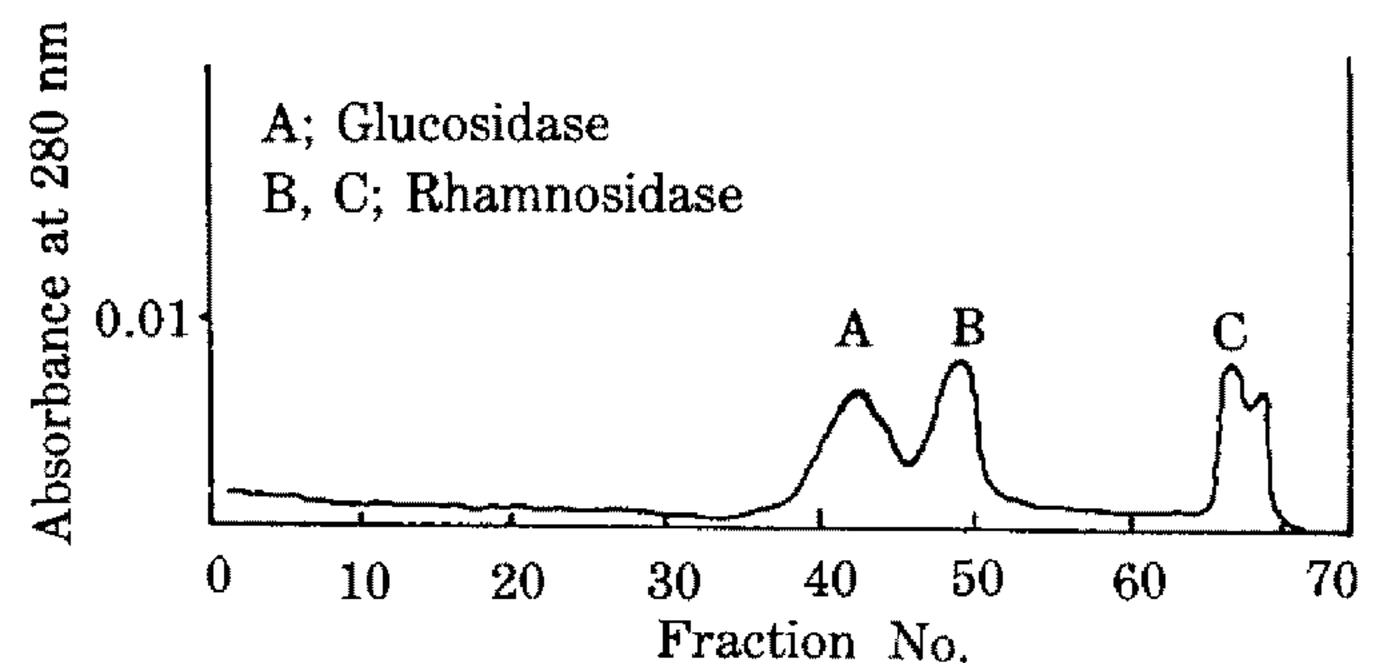


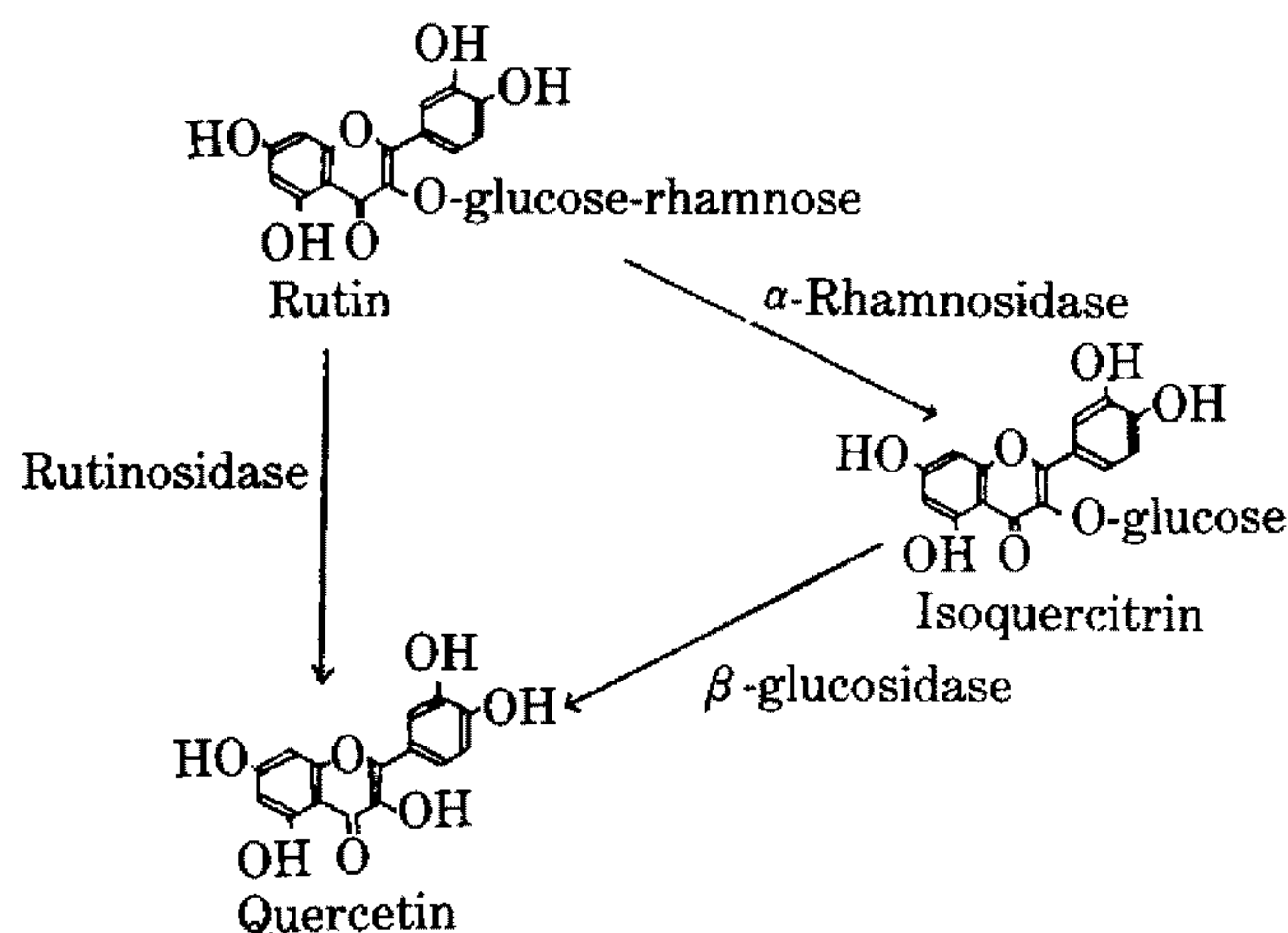
Fig. 2. Chromatography of rutin or isoquercitrin-hydrolysing enzymes on Toyopearl HW55S .

Enzyme purification

The procedure for the purification of the glycoside hydrolysing enzymes from *Arthrobacter* sp. MT-57 is provided in Fig. 1. In Butyl-Toyopearl 650 M column chromatography, Fraction I and Fraction II which eluted with a linear gradient of 12-8% and 5-2% $(NH_4)_2$ SO_4 had enzyme activities, respectively. Fraction I was confirmed containing rutinosidase and had capability of degrading rutin to rutinose and quercetin, these products were detected by TLC. Using TLC, rhamnose, glucose, isoquercetin and quercetin were found in the reaction mixture of the rutin degradation by the Fraction II. It was suggested that in the Fraction II contained rhamnosidase and glucosidase. As shown in Fig. 2, Fraction II had three protein peaks (fraction A, B and C) in Toyopearl HW55S column chromatography. Fraction B and C were confirmed containing α-rhamnosidase and had capability of degrading rutin to rhamnose and isoquercetin. Fraction A was confirmed containing β-glucosidase and had capability of degrading isoquercetin to glucose and quercetin. These above results suggested that the related glycoside hydrolysing enzymes to rutin degradation are α-rhamnosidases, β-glucosidase and rutinosidase as shown in Scheme 1. In the further study, the procedure for the purification of the rutinosidase among the related glycoside hydrolysing enzymes is summarized in Table 1. The rutinosidase was found and have been purified to homogeneity (69-fold) after DEAE-Cellulose DE52 fractionation. The purified enzymes migrated as a single protein band when subjected to electrophoresis on polyacrylamide gel.

Molecular weight

A molecular weight of 40,000 was found on gel filtration on Toyopearl HW50S (Fig. 3). The subunit molecular weight of the enzyme was determined by the method of Weber and Osborn. One protein band



Scheme 1. Proposed mechanism for the glucoside hydrolysing enzymes of rutin.

(42,000) was obtained on SDS-gel electrophoresis (Fig. 4). These results suggested that the enzyme is monomeric.

Optimum pH

The enzyme showed apparent maximum activity at pH 7.5 in potassium phosphate buffer. But in succinic acid-NaOH buffer (pH 6.0), the enzyme activity was 50% less than the maximum activity in potassium phosphate buffer (Fig. 5).

pH stability

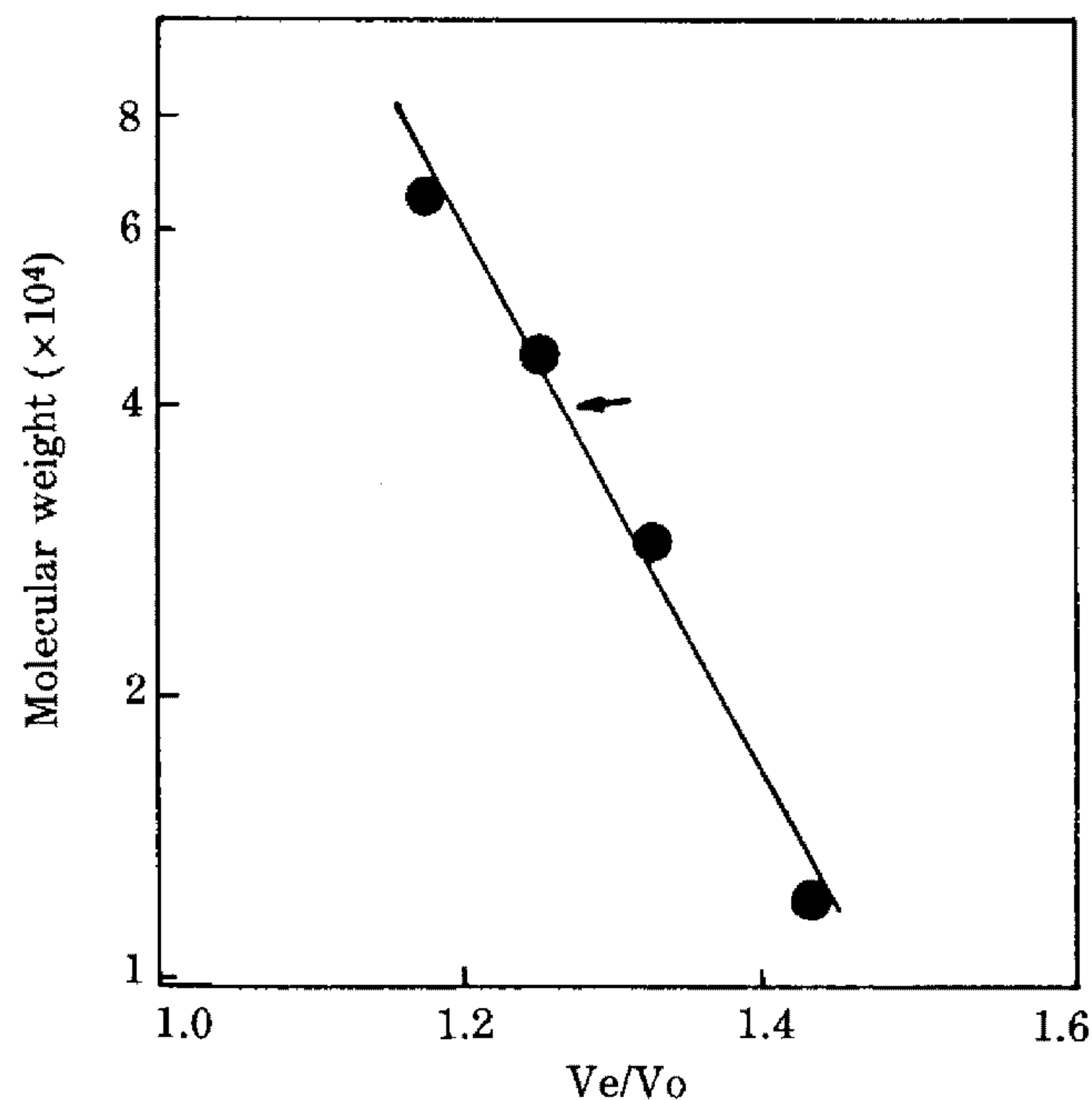


Fig. 3. Estimation of the molecular weight of the rutinosidase by gel filteration on a Toyopearl HW50S column.

The enzyme solution was put on the Toyopearl HW50S column equilibrated with 50 mM potassium phosphate buffer (pH 7.0). The column size was 4.4×71.5 cm. The void volume of the column was determined with blue dextran. The proteins used in this experiment and their molecular weights were as follows; cytochrome C from horse heart (12,400), carbonic anhydrase from bovine erythrocytes (29,000), egg albumin (45,000), and albumin from bovine serum (66,000).

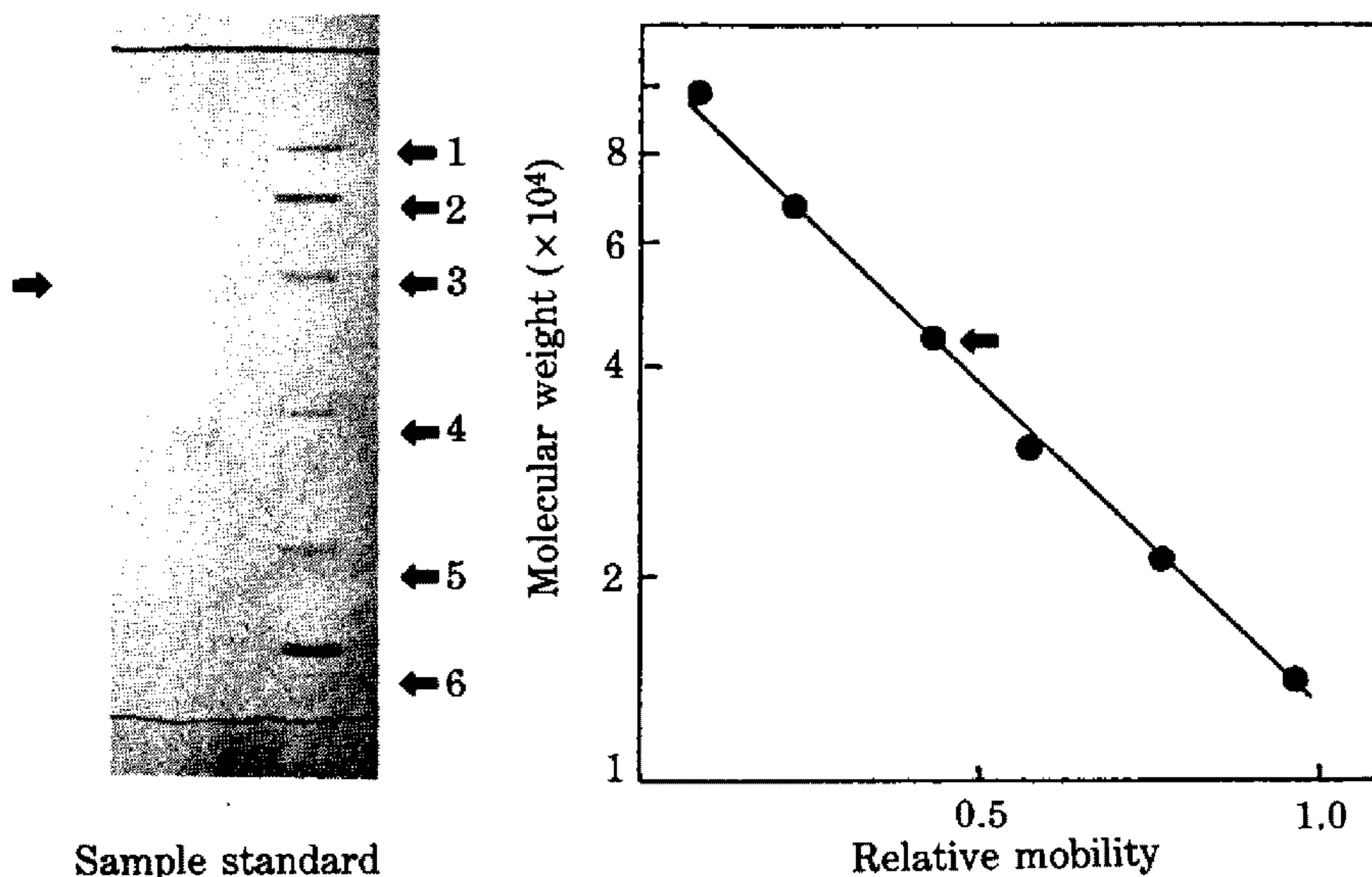


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and molecular weight determination of the purified rutinosidase.

The enzyme was subjected to SDS-polyacrylamide gel electrophoresis after treatment with mercaptoethanol. The standard marker proteins used for calibration purposes were: 1, rabbit muscle phosphorylase b (97,400); 2, bovine serum albumin (66,200); 3, hen egg white ovalbumin (42,700); 4, bovine carbonic anhydrase (31,000); 5, soybean trypsin inhibitor (21,500); and 6, hen egg white lysozyme (14,400).

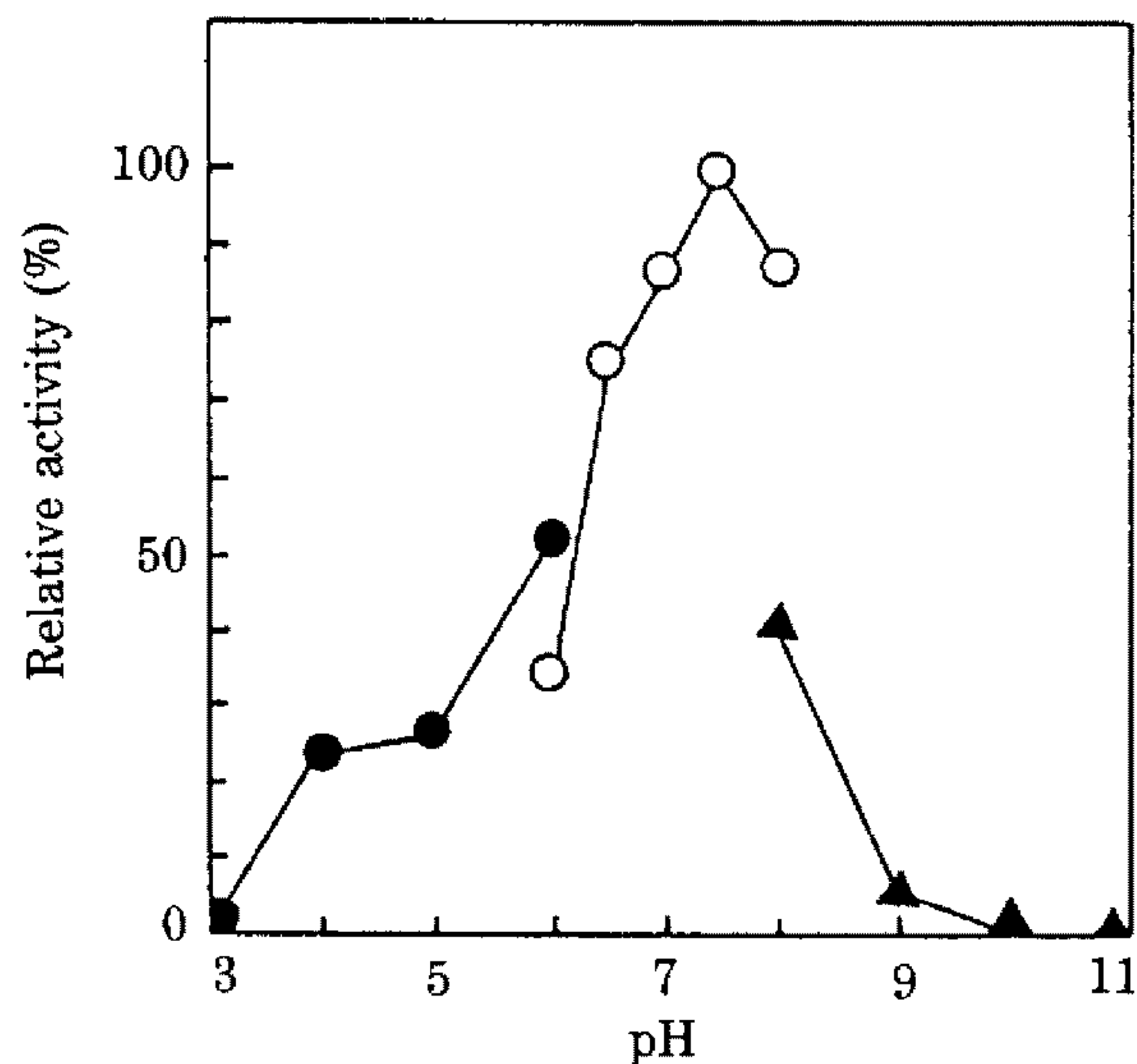


Fig. 5. pH optimum of the rutinosidase.
The pH was adjusted with the following buffer system: -●-, 50 mM succinic acid-NaOH, pH 3.0-6.0; -○-, 50 mM potassium phosphate-NaOH, pH 6.0-8.0; -▲-, 50 mM boric acid-NaOH, pH 8.0-11.0. The enzyme assay was measured spectrophotometically at 450 nm by the formation of quercetin from rutin after incubation with the rutin (0.1 μ mol) in buffers of various pHs for 30 min at 37°C. The ratios of the formation of quercetin per minute were calculated and compared as the relative activity.

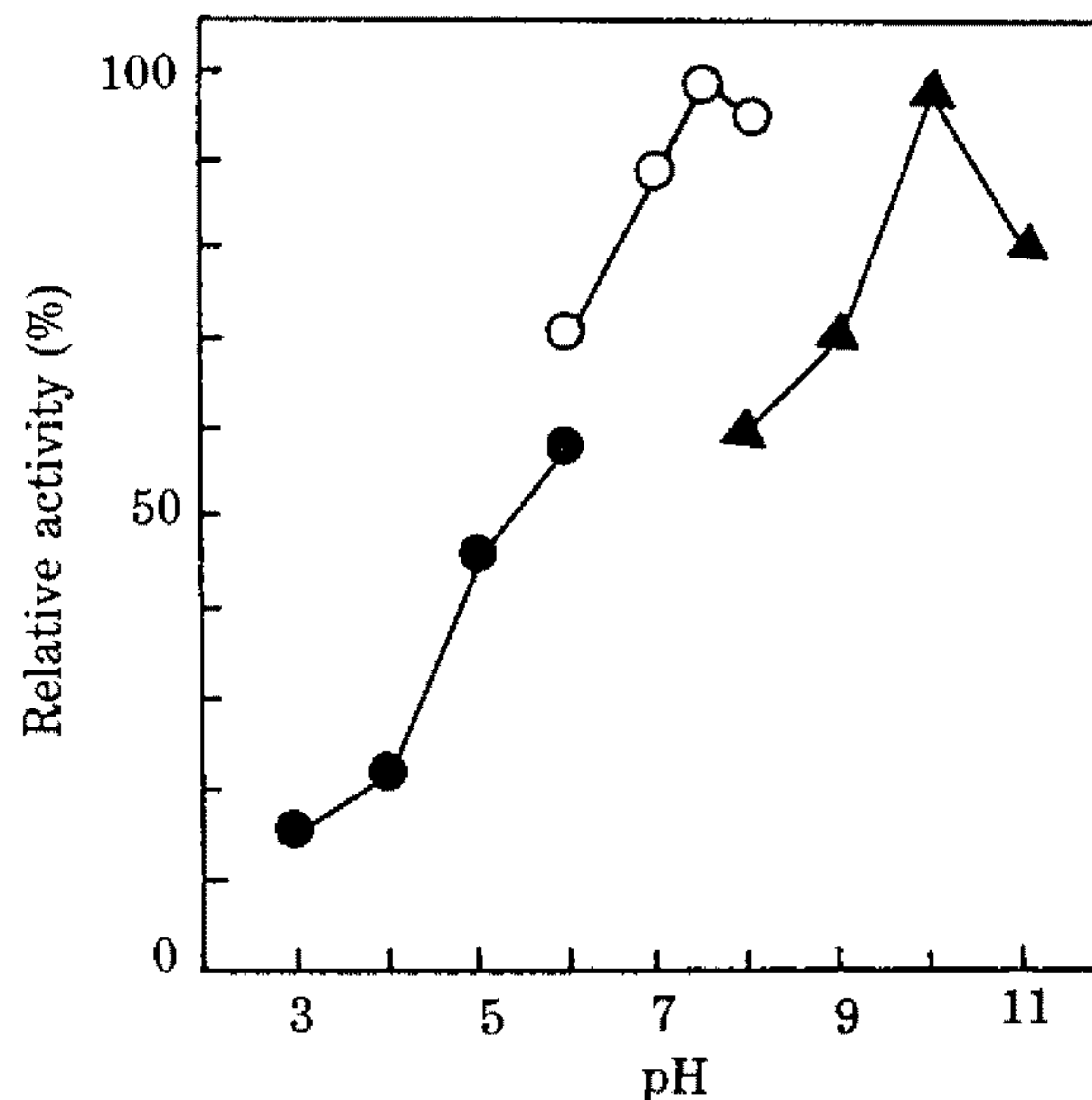


Fig. 6. pH stability of the rutinosidase.
The enzyme was suspended in solutions of various pHs for 20 min at 37°C, and then the residual enzyme activity was assayed at pH 7.0 by the same enzyme assay method in the pH optimum measurement.

As shown in Fig. 6, the enzyme was relatively stable in alkaline solution.

Effects of temperature on the activity and stability of rutinosidase

As shown in Fig. 7, the rutinosidase activity was measured at various temperatures from 20 to 70°C for a 20 min reaction. The maximum rutinosidase activity was observed at 45°C. The thermostabilities of the enzyme was investigated by measuring the residual activities after leaving the enzyme at various temperatures between 20°C and 70°C for 20 min. The rutinosidase was stable up to 50°C.

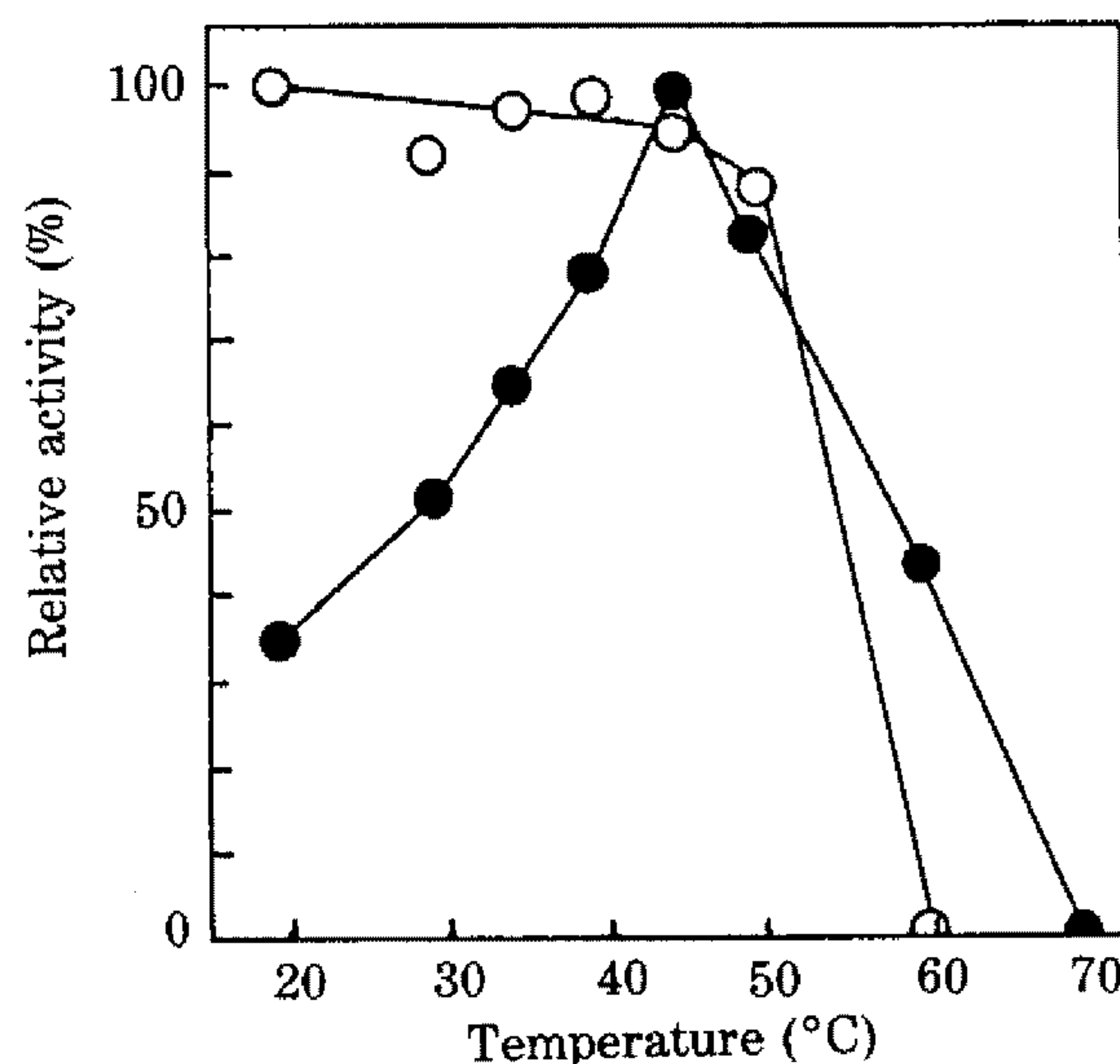


Fig. 7. Effects of temperature on the activity (-●-) and stability (-○-) of the rutinosidase.
The reaction mixture consisting of 0.9 m*l* of 0.1 μmol of rutin in 50 mM potassium phosphate buffer (pH 7.0) and 0.1 m*l* of each purified enzyme solution was incubated at each temperature for 20 min. The enzyme assay was measured spectrophotometrically at 450 nm by the formation of quercetin from rutin.

Enzyme kinetics

The Michaelis constant for rutin was determined with the purified enzyme in 50 mM potassium phosphate-NaOH buffer (pH 7.0). Measurement of the rates of quercetin formation with different concentrations of rutin revealed a K_m of 0.5 μM.

Effects of some reagents and metal ions on the enzyme activity

The effects of inhibitors are summarized in Table 2. This enzyme was not inhibited by thiol reagents, PMSF and carbonyl agents, suggesting that sulfhydryl group, serine group and carbonyl group involved in the catalytic active center of the enzyme did not significantly influence. The chelating agents such as EDTA, NaN_3 and 8-hydroxyquinoline increased the

Table 2. Effects of some reagents and metal ions on the rutinosidase activity.

Reagents (1 mM)	Relative activity (%)
None	100
Thiol reagents	
DTNB	98
PCMB	73
Indoacetate	104
Indoacetamide	105
NeM	97
Serine inhibitor	
PMSF	101
Chelating agents	
EDTA	108
NaN_3	105
Imidazole	97
8-Hydroxyquinoline	119
Carbonyl agents	
Semicarbazide	109
Hydroxylamide	102
Metal ions	
NaCl	73
$MgCl_2$	91
$CaCl_2$	79
$SrCl_2$	73
$CoCl_2$	0
$NiCl_2$	0.1
$CuCl_2$	0

The reaction for enzyme activity were done 10 mM potassium phosphate buffer (pH 7.0) of the 37°C for 30 min.

activity of the enzyme, suggesting that metal ions may be inhibition of the enzyme activity. $CoCl_2$, $NiCl_2$ and $CuCl_2$ were strongly inhibited the enzyme activity, NaCl, $MgCl_2$, $CaCl_2$, $BaCl_2$ and $SrCl_2$ caused partial inhibition of the enzyme activity.

Substrate specificity

. Some flavonoid compounds were tested to examine the substrate specificity of the enzyme (Table 3). The reaction mixture (1 ml), consisting of 0.1 ml enzyme solution and 0.9 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 μmol each substrate, was incubated at 37°C for 7 hr. The enzyme assay was measured of reducing sugar cleaved from rutin after reaction by Somogi-Nelson method at 660 nm. The rutinosidase was active only on rutin among flavonoid compounds contain rutinose, suggesting that enzyme had high substrate specificity in the rutinoside. Rutinosidase was active on flavonoid compounds contain glucose such as isoquercitrin, prunin, and nitrophenyl- β-glucoside, but was not active on quercitrin contain rhamnose. It suggesting that enzyme was active on the glucoside, but was not active on rhamnoside.

Table 3. Substrate specificity of the purified rutinosidase of *Arthrobacter* sp. MT-57.

Substrate	Enzyme activity
Rutinoside	
Rutin	+
Naringen	−
Hesperidin	−
Diosmin	−
Glucoside	
Isoquercitrin	+
Prunin	+
Slicin	−
Nitrophenyl- β-glucoside	+
Rhamnoside	
Quercitrin	−
Galactoside	
Nitrophenyl- β-galactoside	+

요 약

토양으로부터 rutin을 유일한 탄소원으로 생육하는 세균을 분리하여 그 중 rutin 가수분해활성이 높은 MT-57 균을 *Arthrobacter* sp.로 동정하였다. *Arthrobacter* sp. MT-57에서 수 종류의 rutin 가수분해효소가 확인되었으며 이들 중 rutinosidase를 전기영동상 단일한 효소 단백질까지 정제하였다. Rutinosidase의 분자량은 SDS page와 gel filtration으로부터 42,000의 단량체로 추정되었다. 효소반응의 최적 pH와 온도는 pH7.5와 45℃였으며, 20분간 열처리하였을 때 50℃까지 안정하였다.

효소의 rutin에 대한 K_m치는 0.5 μM 이었다. 효소활성은 EDTA, NaN_3, 8-hydroxyquinoline 같은 chelating agent에 의해서는 증가되었으나 Co^{2+}, Ni^{2+}, Cu^{2+}에 의해서는 저해되었다. 또한 본 효소는 rutinoside에 대한 기질특이성이 매우 높은 것으로 나타났다.

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