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Salicylurate-Hydrolyzing Enzyme from Intestinal Bacterium in Rabbit: Purification and Characterization

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An enzyme which hydrolyzes salicylurate (salicyluric acid) into salicylic acid and glycine was found in a cell-free extract of an intestinal bacterium in rabbit. This enzyme, tentatively named salicylurate-hydrolyzing enzyme, was found to be membrane-bound and was purified from the extract by ammonium sulfate fractionation and column chromatography. Two protein fractions with the enzyme activity were observed on diethylaminoethyl-Toyopearl. The first peak (enzyme I) was further purified by chromatography on carboxymethyl-cellulose and on hydroxyapatite, and its enzymatic properties were characterized. The isoelectric point was 8.7, and the molecular weight was estimated to be 170000 by gel filtration on Sephadex G-150 and 27000 by sodium dodecyl sulfate gel electrophoresis, suggesting that the enzyme exists as a hexamer. The enzyme was strongly inhibited by *p*-chloromercuribenzoate (PCMB), Hg^{2+} and *o*-phenanthroline. The activities lost on incubation with PCMB and *o*-phenanthroline were restored by the addition of 2-mercaptoethanol and Zn^{2+}, respectively. The enzyme catalyzed hydrolysis of *N*-benzoyl (Bz) amino acids and their derivatives, while it was inert toward *n*-benzoyl-protected peptides such as *p*-HO–Bz–Gly–Gly and Bz–Gly–L-His–L-Leu. We conclude that salicylurate hydrolase presented here is a kind of hippurate hydrolase (EC 3.5.1.32).

Keywords——salicylurate-hydrolyzing enzyme; rabbit intestinal bacterium; *N*-benzoylglycine amidohydrolase; hippurate hydrolase; salicyluric acid

The glycine-conjugation pathway is common in the metabolism of many phenyl carboxylic acids in various species.[1] On the other hand, the removal of the glycine moiety from glycine conjugates by mammalian intestinal flora has also been reported: hippuric acid by enterococci,[2] *p*-aminohippuric acid by rat cecal microorganisms,[3] *p*-aminohippuric acid and *p*-acetylaminohippuric acid in the gastrointestinal tract of man.[4] In addition, the conversion of salicylurate to salicylic acid was demonstrated in the isolated perfused rat kidney.[5] Boxenbaum *et al.*[6] demonstrated that salicylurate undergoes intestinal microbial metabolism to salicylic acid prior to absorption in healthy subjects and suggested the usefulness of salicylurate as a prodrug, but the details of enzymatic hydrolysis of salicylurate by intestinal microorganisms have remained obscure. Recently, Shibasaki *et al.*[7–9] reported the inability of antibiotic-treated rabbits to form salicylic acid from salicylurate: salicylic acid was not formed by the feces from rabbits treated with the antibiotic, whereas normal feces showed a high activity to hydrolyze salicylurate *in vitro*. Major salicylurate-hydrolyzing activity was observed in the cecum of the intestinal tract.

On the other hand, there are several reports on hippurate hydrolase (EC 3.5.1.32). This enzyme is distributed in various species of microorganisms,[10–20] and has been purified to homogeneity from *Pseudomonas putida* C692-3,[21] *Corynebacterium equi* H-7,[22] and *Streptococcus faecalis* R ATCC 8043.[23,24] These enzymes are specific for *N*-benzoyl amino acids, being preferentially active toward glycine- and/or L-alanine- derivatives.

Our present investigation deals with the purification and characterization of salicylurate-

hydrolyzing enzyme from an intestinal bacterium isolated from rabbit feces, and compares its enzymatic and physicochemical properties with those of the enzymes from other microorganisms.

Materials and Methods

Materials——The following materials were purchased from the source indicated in parentheses: diethylaminoethyl (DEAE) Toyopearl (Toyo Soda Kogyo Co.)., carboxymethyl (CM)-cellulose (Brown Co.), Sephadex G-150, carrier ampholyte, and protein markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Pharmacia Fine Chemicals), *o*-phenanthroline, and *p*-chloromercuribenzoate (PCMB) (Nakarai Chemical Co.), salicylurate and hippurate (Sigma Chemical Co., U.S.A.), Z–Gly–Phe and Bz–Gly–His–Leu (Peptide Institute Inc., Minoh). Hydroxyapatite was prepared as described by Bernardi.[25] *p*-Hydroxybenzoylglycine(*p*-HO–Bz–Gly) was synthesized with *p*-acetoxybenzoate as a starting material by the method of Detar and Silverstein.[26]

The other Bz-amino acids and their derivatives were generous gifts from Dr. Kazuo Ueda, Nagasaki University, Faculty of Liberal Arts.

Assay of Enzyme Activity——The activity of salicylurate-hydrolyzing enzyme was assayed by two methods;

(A) Enzyme activity toward *p*-HO–Bz–Gly was measured by the method of Saruta *et al.*[27] The reaction mixture contained 1 μmol of substrate, 1.25 μmol of 4-amino-antipyrine, and 100 μl of enzyme in 1.5 ml of 25 mM wide range buffer, pH 8.0 (25 mmol barbital, citric acid, potassium dihydrogen phosphate, boric acid). The solution was incubated at 37 °C for 10 min, and the reaction was terminated by adding 0.5 ml of 5 mM sodium periodate. After the solution had been left to stand at 37 °C for 15 min, the absorbance was measured at 500 nm. One unit of the activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol of *p*-hydroxybenzoate per min under the conditions used.

(B) Enzyme activity toward the other substrates was measured by the ninhydrin method. The reaction mixture contained 1 mM substrate and enzyme in a total volume of 0.2 ml. After incubation for 10 min at 37 °C, the enzyme reaction was stopped with 0.2 ml of 3 M acetate buffer, pH 5.0, and the amino acid liberated was measured by the ninhydrin method. The K_m values were calculated from Lineweaver–Burk plots.

The concentration of the enzyme protein was estimated spectrophotometrically by assuming that $A^{1\%}_{1cm}$ at 280 nm is 10, and the specific activity was expressed as the enzyme activity units per mg protein.

Screening of Microorganisms for Salicylurate-Hydrolyzing Enzyme Production——Microorganisms isolated from a fresh rabbit fecal pellet were cultivated on bouillon medium (1% meat extract, 1% polypepton and 0.5% NaCl, pH 7.2), and enzyme production was checked by fluorescence analysis of salicylic acid liberated by the enzyme reaction using salicylurate (*o*-HO–Bz–Gly) as the substrate; 1 ml of cell suspension was added to a tube containing 1 mg of salicylurate in 9 ml of 0.1 M sodium phosphate buffer, pH 7.6. After incubation for 30 min at 37 °C, salicylic acid produced by the enzyme reaction was extracted with 20 ml of carbon tetrachloride. Salicylic acid was transferred to 50 mM glycine buffer, pH 11, from the carbon tetrachloride layer. Fluorescence intensity due to salicylic acid was recorded at the excitation and emission wavelengths of 300 nm and 410 nm, respectively.

Of 250 colonies examined, several colonies were found to show hydrolytic activity toward salicylurate. These colonies were also active toward *p*-HO–Bz–Gly and this activity in each colony was approximately in parallel to that of salicylurate-hydrolyzing enzyme. Thus, the activity was monitored by using *p*-HO–Bz–Gly as the substrate in subsequent experiments.

A gram-positive bacterium which showed the highest activities toward both salicylurate and *p*-HO–Bz–Gly was chosen for production and purification of the enzyme. It was grown aerobically on 300 ml of the preculture bouillon medium (pH 7.2) at 30 °C for 22 h and transferred to an 8 l Microferm Fermentor (New Brunswick) containing buillon medium (pH 7.5), then cultivated for 13 h at 30 °C with aeration of 10 l/min and agitation of 200 rpm. The cells were harvested by centrifugation (8000 rpm for 20 min), and washed twice with 20 mM sodium phosphate buffer, pH 7.0.

Purification of the Enzyme——The cells suspended in 20 mM sodium phosphate buffer, pH 7.0, were disrupted for 20 min with a Dyno-Mill (Willy A. Bachofen), mixed with 1% Triton X-100 as a surface-active reagent, and then treated for 10 min with a Sonifier Cell Disruptor (Branson). The turbid mixture was centrifuged at 12000 rpm for 20 min and protamine sulfate (final concentration, 0.2%) was added to the supernatant to precipitate viscous materials. After centrifugation (12000 rpm for 20 min), the cell-free extract was fractionated with ammonium sulfate (35—70% saturation). The precipitate was dissolved in a small volume of the same buffer, desalted by gel filtration on Sephadex G-25 and then applied to a column (4.3 × 30 cm) of DEAE-Toyopearl equilibrated with the same buffer. The first enzyme peak (enzyme I) appeared in the void fraction and the second enzyme peak (enzyme II) appeared in the 0.3 M NaCl eluate. The former enzyme solution was applied to a column (2.7 × 15 cm) of CM-cellulose equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The adsorbed enzyme was eluted by a linear gradient of NaCl concentration from 0 to 0.4 M. The active fractions were combined and dialyzed against the buffer. Then, the dialyzate was applied to a column (1.5 × 4 cm) of hydroxyapatite equilibrated with 10 mM phosphate buffer, pH 7.0.

The enzyme was eluted with a linear gradient of phosphate concentration from 10 to 800 mM. The active fractions were combined, concentrated by ultrafiltration with an Amicon PM-10 equipment and kept at −20°C until use.

Estimation of Molecular Weight——The molecular weight was estimated by gel filtration on a Sephadex G-150 column (1.8 × 120 cm) according to Andrews[28] using 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 M KCl. Catalase from bovine liver (MW 240000), creatininase from *Pseudomonas putida* (MW 175000), alcohol dehydrogenase from yeast (MW 141000), prolyl endopeptidase from bovine brain (MW 76000) and trypsin from bovine pancreas (MW 24000) were used as reference proteins. SDS-PAGE as described by Weber and Osborn[29] was also used for estimation of subunit molecular weight. Phosphorylase b from rabbit muscle (MW 94000), bovine serum albumin (MW 67000), egg white ovalbumin (MW 43000), carbonic anhydrase from bovine erythrocytes (MW 30000), soybean trypsin inhibitor (MW 20100) and α-lactalbumin from bovine milk (MW 14400) were used as the standard marker proteins.

Disc Gel Electrophoresis and Isoelectric Focusing——The purity of the preparation was examined by disc gel electrophoresis based on the method of Davis.[30] A 7.5% separation gel in β-alanine–acetate buffer, pH 4.5, and methylene green as a running marker were used. After gel electrophoresis, one of the gels was stained with 1% Amidoschwarz 10B for 30 min and destained in 7% acetic acid overnight. The other gel was used to evaluate the location of enzymatic activity. The gel was cut at intervals of 5 mm, and the slices were reacted with a mixture containing 2 μmol of *p*-HO–Bz–Gly and 7.5 μmol wide range buffer, pH 8.0, overnight. To determine the isoelectric point, the enzyme was applied to an isoelectric focusing column according to the method of Vesterberg and Svensson[31] using a carrier ampholyte in the pH range of 3.5 and 10.0 and with sucrose to form a density gradient from 0 to 50%. The column was then subjected to isoelectric focusing at 300 V for 60 h at 4°C.

Results and Discussion

Screening of Microorganisms for Salicylurate-Hydrolyzing Enzyme Production

After screening of more than 200 colonies from rabbit feces, we chose one colony with potent activity toward salicylurate. The taxonomic characteristics of this strain were not tested in detail, except that the strain was positive on Gram staining, and was in a monococcus or diplococcus form. It seems likely that this strain belongs to enterococci which are known to be gram-positive bacteria capable of hydrolyzing sodium hippurate into benzoic acid and glycine.[2] The enzyme activity was not detected in the culture filtrate. Although Rohr[10] reported that hippurate hydrolase was induced by benzoic acid, the intestinal bacterium was found to produce the enzyme without addition of any inducer.

Purification of Salicylurate-Hydrolyzing Enzyme

The cell-free enzyme preparation was obtained by means of physico-chemical treatments as described. After removal of nucleic acid and other impurities and fractionation with ammonium sulfate from 35 to 70% saturation, the crude enzyme was prepared. Two peaks of the enzyme activity were found on DEAE-Toyopearl chromatography (Fig. 1). The enzyme which appeared in the void fraction (enzyme I) was further purified by using columns of CM-cellulose and hydroxyapatite. By these procedures, enzyme I was purified about 600-fold with an activity recovery of 1% (Table I). The purity of the enzyme preparation was checked by

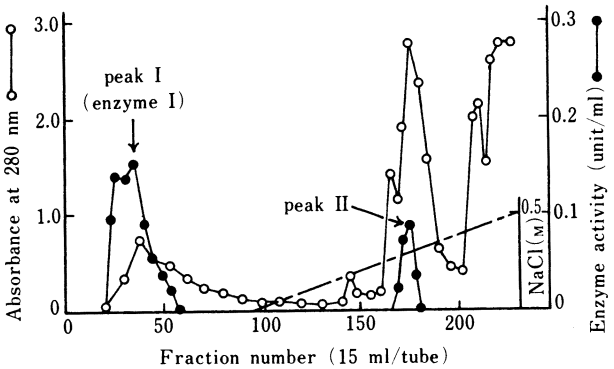

Fig. 1. DEAE-Toyopearl Chromatography of Salicylurate-Hydrolyzing Enzyme

Crude enzyme solution (protein, 5600 mg; activity, 140 units in a total volume of 310 ml) was applied to the column (4.3 × 30 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.0.

TABLE I. Summary of Purification Procedure of Enzyme I

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (units/mg)	Purification ratio (fold)	Yield (%)
Dyno-Mill	45410	2520	0.056	1.0	100
Ultrasonication	23830	1650	0.069	1.2	65
Protamine sulfate	23040	1100	0.048	0.9	44
Ammonium sulfate fractionation	16280	939	0.058	1.0	37
DEAE-Toyopearl	962	451	0.49	8.7	18
CM-cellulose	7	182	26	464	7
Hydroxyapatite	1	31	31	553	1

disc gel electrophoresis. The final preparation was still heterogeneous electophoretically, being contaminated with a minor impurity. The enzyme activity coincided with the major protein band.

The two peaks separated by ion exchange chromatography resemble each other in optimal pH, pH stability, and isoelectric point, but had different molecular weights. When the enzyme of high molecular mass (enzyme II) was exposed to ultrasonication treatment for 30 min and then applied to a column of Sepharose 6B, the enzyme II was eluted at the same position as that of enzyme I. Therefore, the active principles of both peaks seem to be identical or similar. These enzymes are probably membrane-bound enzymes, because the treatment with Triton X-100 was necessary for extraction of the enzymes from cell debris.

Estimation of Molecular Weight and Isoelectric Point

The molecular weight of enzyme I was estimated to be 170000 by gel filtration on Sephadex G-150 and 27000 by SDS-PAGE (Fig. 2), suggesting that the enzyme consists of six subunits identical in molecular mass. The isoelectric point was about 8.7 as checked by the isoelectrofocusing method. The pI value of enzyme I is higher than those of other microbial hippurate hydrolases.[21-23]

There is a variety of subunit structures in the microbial benzoylglycine amidohydrolases.[21-23] The subunit structure of the intestinal bacterium enzyme I is identical to that of the *Corynebacterium* enzyme. Our enzyme I and the enzyme from *C. equi* are composed of six subunits identical in molecular weight, while the enzyme from *P. putida* and the enzyme from *S. faecalis*, which is a strain of intestinal bacteria, consist of four and eight identical subunits, respectively.

Some Enzymatic Properties

The optima of pH and temperature for the activity of the enzyme were 9.0 and 45 °C, respectively, using *p*-HO–Bz–Gly as the substrate. More than 90% of the original activity remained after incubation for 17 h at 25 °C between pH 7.0 and 10.0. Incubation at pH 7.0 and 55 °C for 30 min led to about 50% loss of the activity.

The enzyme was strongly inhibited by 1 mM PCMB, *o*-phenanthroline and Hg^{2+} but was not affected by 1 mM ethylenediaminetetraacetate, iodoacetic acid or 5,5′-dithio-bis-(2-nitrobenzoate) (Table II). The enzyme inactivated with PCMB was reactivated by the addition of 2-mercaptoethanol and dithiothreitol (Fig. 3). The enzyme inactivated with *o*-phenanthroline was completely reactivated by the addition of Zn^{2+} (Fig. 4). These results suggest that zinc ion and sulfhydryl group(s) participate in the catalytic mechanism of the enzyme. However, it remains to be elucidated whether a sulfhydryl group is directly involved in the catalytic reaction, since there is a possibility that introduction of bulky group such as PCMB into cysteinyl residue(s) located near the active site induces steric hindrance in the

TABLE II. Effects of Various Chemicals and Metal Ions on Enzyme Activity

Chemical (1 mM)	Relative activity (%)	Chemical (1 mM)	Relative activity (%)
None	100	Ca^{2+}	95
o-Phenanthroline	12	Mn^{2+}	150
EDTA	98	Co^{2+}	96
8-Hydroxyquinoline	54	Cu^{2+}	109
α,α′-Dipyridyl	96	Zn^{2+}	85
PCMB	2	Fe^{3+}	53
NEM	107	Hg^{2+}	2
Iodoacetic acid	112	Cd^{2+}	80
DTNB	87	Sn^{2+}	35
Na_2SO_4	101	Ni^{2+}	82
PMSF (0.5 mM)	102	Mg^{2+}	91
STI	110		
DFP	134		
Pepstatin	121		

The reaction mixture contained 0.04 unit of the enzyme in phosphate buffer, pH 7.0, and the indicated amounts of chemicals and metal ions. After preincubation for 10 min at 37 °C, the enzyme activity was assayed by the standard method using *p*-HO-Bz-Gly as described in Materials and Methods. EDTA, ethylenediaminetetraacetate; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); PMSF, phenylmethanesulfonyl fluoride; STI, soybean trypsin inhibitor; DFP, diisopropyl fluorophosphate.

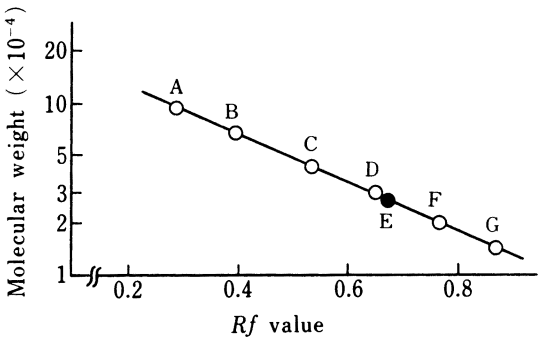

Fig. 2. Estimation of Molecular Weight of Salicylurate-Hydrolyzing Enzyme by SDS-Polyacrylamide Gel Electrophoresis

See the text for experimental details. (A) Phosphorylase b, 94000; (B) bovine serum albumin, 67000; (C) ovalbumin, 43000; (D) carbonic anhydrase, 30000; (E) salicylurate-hydrolyzing enzyme; (F) soybean trypsin inhibitor, 20100; (G) α-lactalbumin, 14400.

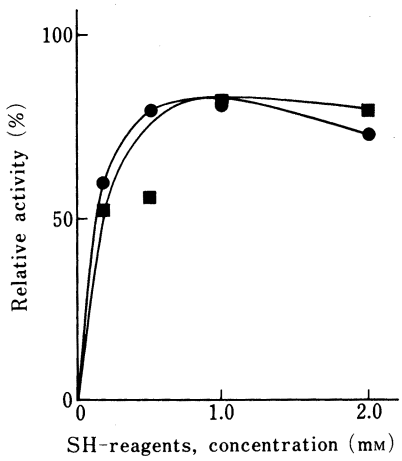

Fig. 3. Reactivation of PCMB-Inactivated Enzyme I by Incubation with Sulfhydryl Reagents

●, 2-mercaptoethanol; ■, dithiothreitol. The reaction mixture, containing 5 ml of 2 mM PCMB and 5 ml of the enzyme (0.5 unit), was preincubated at pH 8.0, for 10 min at 37 °C, and then dialyzed against 20 mM sodium phosphate buffer, pH 7.0, to remove PCMB. To 0.45 ml of the dialyzate was added 0.05 ml of various concentrations of sulfhydryl reagent. After incubation for 10 min at 37 °C, the enzyme activity was assayed by the ninhydrin method.

enzyme–substrate interaction. Furthermore, if PCMB reacts with cysteine residue(s) coordinated to the active site metal ion, the enzyme might irreversibly lose its activity.

Substrate Specificity

The substrate specificity of enzyme I was investigated (Table III). The enzyme was active

TABLE III. Substrate Specificity of Enzyme I

Substrate	Relative activity (%)	K_m value (mM)
p-HO-Bz-Gly[a]	100	1.4
p-Cl-Bz-Gly[b]	270	2.5
p-NO_2-Bz-Gly[b]	210	2.0
Bz-Gly[b]	156	1.0
p-CH_3O-Bz-Gly[b]	106	0.5
Z-Gly[b]	2	
Boc-Gly[b]	1	
Acetyl-Gly[b]	0	
o-HO-Bz-Gly[b]	12	0.2
p-HO-Bz-L-Ala[a]	72	0.6
p-HO-Bz-*β*-Ala[a]	0	
p-HO-Bz-L-Glu[a]	21	2.5
p-HO-Bz-Gly-Gly[a]	0	
Bz-Gly-L-His-L-Leu[b]	0	
Z-Gly-L-Phe[b]	0	

a) The enzyme activities were assayed by the standard assay method using *p*-HO-Bz-Gly as described in Materials and Methods. *b*) The enzyme activities were assayed by the ninhydrin method as described in Materials and Methods.

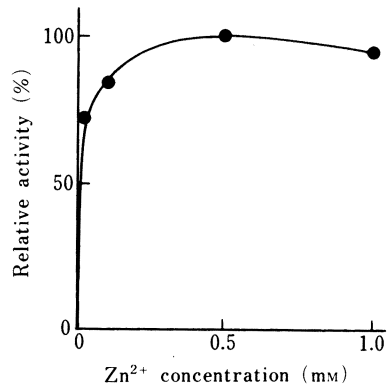

Fig. 4. Reactivation of *o*-Phenanthroline-Inactivated Enzyme I by Incubation with Zinc Ion

The reaction mixture, containing 0.1 ml of 10 mM *o*-phenanthroline and 0.9 ml of the enzyme (0.14 unit), was preincubated at pH 7.0, for 10 min at 37 °C. One-tenth milliliter aliquots of the solution were mixed with 0.5 ml of various concentrations of $ZnCl_2$. Each mixture was incubated for 10 min at 37 °C, and the enzyme activity was assayed by the standard assay method.

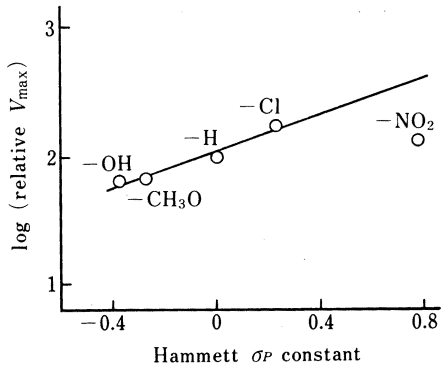

Fig. 5. Correlation between Hammett's σP Constant and Relative V_{max}

The enzyme activity was assayed by the ninhydrin method. The V_{max} values were calculated by means of a Lineweaver-Burk plot and were expressed relative to that of *N*-benzoylglycine.

toward Bz-Gly, *p*-substituted Bz-Gly(s), *p*-HO-Bz-L-Ala, *p*-HO-Bz-L-Glu, and *o*-HO-Bz-Gly in decreasing order. The apparent K_m values for Bz-Gly, *p*-HO-Bz-Gly, *p*-HO-Bz-L-Ala, *o*-HO-Bz-Gly were calculated to be 1.0, 1.4, 0.6 and 0.2 mM, respectively. The ability of the enzyme to hydrolyze *N*-acyl compounds with bulky substituents such as benzyloxycarbonyl (Z-) and *tert*-butoxycarbonyl (Boc-) groups was negligibly low. The enzyme was also inert toward acetyl-Gly and *p*-HO-Bz-*β*-Ala. A variety of *N*-benzoyl peptides such as *p*-HO-Bz-Gly-Gly, Z-Gly-L-Phe and Bz-Gly-L-Leu were not susceptible to the enzyme. These results indicate that the enzyme recognizes the benzoyl moiety as an acyl group and amino acids such as glycine, L-alanine, and L-glutamic acid. Thus, the enzyme is concluded to be a typical hippurate hydrolase. The effect of the *p*-substituted group on the hydrolysis of the acyl bond of *N*-benzoylglycine was examined. The apparent reaction rate was in the following order: *p*-Cl > *p*-NO_2 > H > *p*-CH_3O > *p*-HO. Electron-attracting groups such as Cl and NO_2 increased the rate, but electron-donating groups such as CH_3O and HO decreased it. The differences in apparent reaction rate seem to depend on the electronic effects of *p*-substituents on the acyl bonds of these substrates. A linear relationship was observed between the

TABLE IV. Characteristics of *N*-Benzoylglycine Amidohydrolase from Various Strains

Strain	Intestinal bacterium	*P. putida*	*C. equi*	*S. faecalis*
Optimal pH	9.0	7.0—8.0	8.0	6.0
Stable pH	7.0—10.0	6.0—8.0	7.0—8.0	—
Optimal temp. (°C)	45	50	45	35
Thermal stability (°C)	55	50	45	—
Molecular weight	170000	170000	230000	220000
Subunit number	6	4	6	8
Isoelectric point	8.7	4.2	4.6	4.5
K_m (mM)				
p-HO-Bz-Gly	1.4	0.54	3.5	0.1
Bz-Gly	1.0	0.72	6.7	0.18
Inhibitor	OP, PCMB Hg^{2+}	OP, PCMB Hg^{2+}, Cu^{2+}	OP, PCMB Hg^{2+}, Cu^{2+}	PCMB
References	Present work	21	22	23

OP, *o*-phenanthroline; PCMB, *p*-chloromercuribenzoate; *P. putida*, *Pseudomonas putida* C-692-3; *C. equi*, *Corynebacterium equi* H-7; *S. faecalis*, *Streptococcus faecalis* R-ATCC 8043.

Hammett σp constants[32] and logarithm of relative V_{max} of these *p*-substituted substrates (Fig. 5), except that *p*-NO_2-substituent was far from the line, presumably because of its steric hindrance.

Various species of microorganisms[10-24] degrade hippuric acid to benzoic acid and glycine. The enzymatic properties of the present intestinal bacterium were compared with those of the other microorganisms (Table IV). The enzymes resemble each other in enzymatic properties and molecular weight, but salicylurate-hydrolyzing enzyme was localized in the cell membrane and the isoelectric point of the enzyme was quite different from those of the other enzymes. Most of the enzymes from bacteria could hydrolyze *N*-Bz-Gly, *N*-Bz-L-Ala, and *N*-Bz-L-aminobutyric acid but could not act on *N*-acetylamino acids or various *N*-Bz-peptides. These types of the enzymes could be divided into two classes according to their substrate specificity. One type, including the present enzyme, is *N*-benzoylglycine amidohydrolase, which hydrolyzes *N*-Bz-Gly more rapidly, and the other type is *N*-benzoylalanine amidohydrolase, which is more active toward *N*-Bz-L-Ala, as in the case of the enzyme from *C. equi*.

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