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Determination of specific activities and kinetic constants of biotinidase and lipoamidase in LEW rat and *Lactobacillus casei* (*Shirota*)☆

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To remember the kind contribution and encouragement of the Late Hon. Prof. Dr. Kunio Yamauchi (5 April 1928–10 March 2004).

Abstract

Enzyme kinetic parameters, such as K_m, V_{max} (or V), k_{cat}/K_m, and K_i (by biotin or lipoic acid) for biotinidase and lipoamidase were determined in Lewis (LEW) rat and *Lactobacillus casei* (*Shirota*) using fluorimetric high-performance liquid chromatography (HPLC). It was found that the final protein concentration below 0.1 mg/ml is sufficient to obtain linear hydrolytic reaction and to determine the Michaelis–Menten type kinetic parameters (K_m, V, K_i). We applied this HPLC enzyme assay method onto the rat and some bacteria. The highest specific activities (Vs) for biotinidase were found in *Lactobacillus casei* (*Shirota*) and rat kidney. It was also found that the largest K_i by product for biotinidase and lipoamidase were present in the *Lactobacillus casei* (*Shirota*). There has been found specie (between rat and mouse) differences and tissue (organ) differences, together with tissue region differences and sex differences in some tissues. Summary of the distributions of both enzymes in LEW rat was also presented. Therefore, this HPLC determination method for the enzyme kinetic parameters in tissues is expected to be an indispensable tool for the investigation of the various diseases in humans.

Keywords: Enzyme kinetics; Biotinidase; Lipoamidase (lipoyl-X hydrolase); LEW rat; *Lactobacillus casei* (*Shirota*); Tissue homogenate; HPLC-fluorimetric enzyme assay; SEC protein assay

1. Introduction

Biotinidase (EC 3.5.1.12) and lipoamidase (EC number not yet given) are amido-hydrolases (or amidases), which hydrolyze and liberate vitamin of biotin and vitamin-like active substance of lipoic acid from the biotin-amide and lipoic acid-amide substrates, respectively. We studied and developed the precise and convenient determination method to obtain the kinetic parameters of biotinidase and lipoamidase.

Determination of enzyme kinetic parameters, such as K_m, V_{max} (V), and K_i, is essential in the enzyme study, because the enzyme reaction depends on the concentrations of the substrate under the law of mass-reaction as described by Michaelis and Menten [1]. The enzyme in full activity should possess both the

Abbreviations: BAQ, biotinyl-6-aminoquinoline; LAQ, lipoyl-6-aminoquinolin; AQ, 6-aminoquinoline; A_{mo}, affinity; K_{ip}, inhibition constant by the product; K_{is}, inhibition constant by the other substrate; R_{ep}, repulsion; C_{ap}, enzyme capacity; BPAB, biotin 4-amidobenzoic acid (sodium salt); PBS, phosphate-buffered saline; PCMB, *para*-chrolomercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; SEC, size-exclusion chromatography; LEW, Lewis rat

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affinity for the substrate and the repelling power to the product, i.e., the reaction center should be empty for the next substrate molecule to come into the active center.

The affinity for the substrate is usually expressed by the inverse of K_m (Michaelis constant) or k_{cat}/K_m (and we would like to designate this latter affinity parameter as A_{mo} in this text, i.e., *love* or *sincere* in *Latin*). Preliminary studies indicated that the urea-treated lipoamidase of *Lactobacillus casei* (*Shirota*) showed larger K_m and V values than those of the native (non-treated) enzyme. Therefore, A_{mo} was decreased in accordance with the denaturation (or inactivation) of the enzyme.

With respect to the K_{ip} (product inhibition constant), we confirmed the previous findings of Knappe et al. [2] that the K_i (by the presence of biotin) of biotinidase from *Lactobacillus casei* was relatively large (780 μM) as compared with that of porcine kidney biotinidase (93 μM). Therefore, we call this product inhibition constant as K_{ip}, and recognize that this large K_{ip} value is a reflection of the product repelling power from the enzyme. We understand that to study the ability of the enzyme to repel the product should become an important issue in the enzyme kinetic research.

In this study, we exhibited an improved and sensitive determination method of biotinidase and lipoamidase enzyme kinetic parameters by using high-performance liquid chromatography (HPLC) with fluorimetric detection. Sensitive and specific substrates of biotinyl-6-aminoquinoline (BAQ) for biotinidase [3,4] and lipoyl-6-aminoquinoline (LAQ) for lipoamidase [5] were used.

Study on biotinidase and lipoamidase activities at a fixed concentration of substrates in the rat has been previously presented using the wet weight bases except liver [6]. However, comparison of activity becomes meaningful by using the specific activity. Thus, we applied the previously developed sensitive and reliable size-exclusion chromatographic (SEC) protein assay [7–9] in this study. This report, to the best of our knowledge, is the first to describe and summarize widely and quantitatively compare the tissue and bacterial biotinidase and lipoamidase enzyme kinetic parameters. Furthermore, in this report we are the first to mention and discuss about the importance of K_{ip} by the product as the repulsion power of the enzyme against the product.

2. Materials and methods

2.1. Protein determination

The amount of protein was determined by the recently developed size-exclusion chromatography method using bovine serum albumin (BSA) as a standard [7–9]. In order to assess the protein stability of the homogenate samples, monitoring by the size-exclusion chromatographic elution pattern was sometimes performed [10]. SEC is surely a classic method; however, we are the first to use SEC for the protein determination [7–9]; this is a unique SEC method using an extremely short-length column (35 mm long) in order to elute all the proteins at the retention time of the exclusion volume (without separation of proteins). As described in ref. [9] (p. 22), determination of 40 ng of BSA was performed at coefficients of variation (CV) of 2.3% (within-day repeatability). Determination of 2 μg of BSA was performed at CV 1.1% (within-day repeatability) and CV 1.9% (day-to-day repeatability). The two-point linear calibration curve was used [7], and this level of protein determination was about 200 times higher sensitivity than the classic photometric protein assay (BCA method) [8].

2.2. Chemicals and reagents

Biotinyl-6-aminoquinoline (BAQ; *M*r 370.5), biocytin (ε-N-biotinyl-L-lysine; *M*r 372.5), biotin 4-amidobenzoic acid (sodium salt; BPAB; *M*r 385.4), Trizma base, BSA (Cohn fraction V; *M*r 66,000), *para*-chloromercuribenzoate (PCMB), and lysozyme from hen-egg white were purchased from Sigma (St. Louis, MO, USA). D-Biotin, phenylmethanesufonyl fluoride (PMSF), sodium dihydrogenphosphate dihydrate, *ortho*-phosphoric acid (85%), glycerol, EDTA (tetrasodium salt), 2-mercaptoethanol, acetonitrile, methanol, chloroform, and 2-propanol were from Wako (Osaka, Japan). Brij 58 (polyethyleneglycol 1000 monocetylether) and DL-α-lipoic acid were from Nacalai Tesque (Kyoto, Japan). 6-Aminoquinoline (AQ) was from Aldrich, Milwaukee, WI, USA. Lipoyl-6-aminoquinoline (LAQ; *M*r 332.5) was synthesized as previously described [5]. SEC columns of Develosil 50 Diol-5 (35 × 8 mm I.D., 5 nm average pore diameter, 5000 nm average particle size), Develosil 300 Diol-5 (300 × 8 mm I.D., 30 nm average pore diameter, 5000 nm average particle size), and Develosil ODS-10 were from Nomura (Aichi, Japan). Ekicrodisc 13CR and 25 (200 nm average pore diameter) was from Gelman Sciences (Ann Arbor, MI, USA).

2.3. Specimens

2.3.1. Rat tissues

Tissue homegenates from LEW rat (8 weeks of age) were prepared as described in refs. [7] and [10]. The homogenization buffer (1 mM sodium phosphate buffer (pH 7.0, containing 10% v/v glycerol) was prepared as in refs. [7,10], and autoclaved. LEW rats (inbred strain) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). Perfusion of rat liver was performed with 0.9% (154 mM) sodium chloride solution. As shown in Table 1, low concentration of ethyl ether (gas) was not inhibitory to biotinidase; however, bactericidal use of ethanol during skin collection was found to detrimental to the skin biotinidase. Therefore, the rat skins were collected without using ethanol. Suffocation by using dry-ice was not found to be good for measuring the stomach enzymes, and the tissues were collected after anesthetization with ether [7]. In order to perform the subcellular distribution studies of enzymes, tissue and bacterial homogenates were ultracentrifuged at 100,000 × g for 90 min at 4 °C (Beckman L8-M Ultracentrifuge; Rotor Type 35). The supernatant fraction and precipitated membrane fraction (containing nuclear proteins) were obtained. Membrane fractions were dispersed by using Pasteur pipettes (Fisher Scientific, USA), or ultrasonicated for 5 s under the cooling condition. Supernatant and membrane fractions obtained were also stored at −196 °C until use.

Table 1
Inhibition of rat kidney biotinidase by organic solvents[a]

Organic solvent Concentration (%, v/v)	Activity relative to control (%)
Ethyl ether	
0	100
2	109
2.5	104
5	91
10	64
Ethanol	
0	100
0.5	105
1	86
2.5	82
5	59
Methanol–acetonitrile (1:1, v/v) solution (stopping solution)	
0	100
2.5	86
5	73
10	32
15	5
33.3 (stopping condition)[b]	0

[a] Specific activity of male rat kidney homogenate at 20 μM of BAQ was 75.5 pmol/min/mg of protein. Enzyme (0.202 mg/ml) in 0.1 ml of reaction buffer (0.1 M of Tris–HCl buffer (pH 7.0) containing 1 mM of EDTA and 10% (v/v) glycerol) was pre-incubated with respective concentrations (v/v, %) of organic solvents for 15 min at 37 °C. After 15 min, 0.1 ml of substrate containing reaction solution (40 μM of BAQ) was added to start the reaction. Protein concentration at reaction was 0.101 mg/ml, and reaction time was 20 min. Other conditions are as described in Section 2.

[b] Without pre-incubation.

2.3.2. Rodent and human serum

LEW rat (inbred) and Balb/c mouse (inbred) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan), and SD rat (outbred), C57BL/6 mouse (inbred) and ICR mouse (outbred) were from Japan Clea (Tokyo, Japan). Rat and mouse used were 8 weeks of age. Rodent plasma was exsanguinated after anesthetization with ethyl ether, and serum was prepared after centrifugation of the plasma. Human serum was kindly donated by the National Center for Child Health and Development (Tokyo, Japan) and the Saisei-kai Maebashi Hospital (Maebashi, Gunma, Japan). Serum was diluted by the serum dilution buffer (SDB; ref. [8]), about 150-folds (protein concentration less than 1.0 mg/ml), and diluted serum was used within a day.

2.3.3. Lactobacillus casei (Shirota strain and NY1301 strain)

Commercially available beverages were purchased from a food shop. Sixty-five milliliters of Yakult (Yakult Co., Tokyo, Japan) contains ca. 6.5×10^9 cells of *Lactobacillus casei* (*Shirota* strain). One hundred thirty milliliters of Yakult was centrifuged at 26,200 × *g* for 20 min at 2 °C. Precipitated cells were resuspended with 0.1 M sodium phosphate buffer (pH 7.0), and then centrifuged. This washing procedure was repeated. Finally, bacterial cells were resuspended to 50 ml with PBS. 0.1 mg of lysozyme (from hen-egg white) was added to 0.8 ml of cell suspension, and incubated at 37 °C for 5 min. A portion of the mucous bacterial cells (or protoplasts) was then ultra-sonicated, and the homogenate was diluted with the homogenization buffer and filtered by Ekicrodisc 25. This filtered homogenate was stored in liquid nitrogen (at −196 °C) until use.

Lactobacillus casei (*NY1301* strain) was similarly obtained from a beverage of Seikyo Pirukuru (Japan Livelihood Co-operative Association, Shibuya, Tokyo, Japan), and processed as above.

2.3.4. Bacillus natto

Commercially available Japanese soybean food of Natto (Takano Foods Co., Ogawa-town, Ibaragi, Japan) was purchased from a food shop. After mixing the Natto with a pair of chopsticks, *Bacillus natto* was extracted from the mucous material with PBS. The bacterial cells extracted were further washed six times with PBS by centrifugation. Homogenate was prepared by using lysozyme as described above. Protein concentrations of the bacterial homogenates were corrected by the added amount of lysozyme.

2.4. Determination of K_m, V, A_{mo} (k_{cat}/K_m), K_{is} and K_{ip}

K_m and V of biotinidase and lipoamidase were determined by the HPLC-fluorimetric method as follows. This improved method essentially depends on the methods as described previously in refs. [3–5].

Fluorescence detector used was RF-10A$_{XL}$ with a cell temperature controller (at 20 °C; Shimadzu, Kyoto, Japan). One of the product AQ was separated by the ODS column (50 mm × 4.6 mm I.D.; packed with Develosil ODS-10), and detected at the excitation wavelength of 350 nm and the emission wavelength of 550 nm [3–5]. Column temperature was maintained at 8 °C by using CTO-10AC column oven (Shimadzu) with the aid of pre-frozen (−20 °C) cooling bags in order to assay lipoamidase activity reproducibly. Solvent A was 0.1 M sodium phosphate buffer (pH 2.1): methanol = 90:10 (v/v), and solvent B was methanol. The HPLC system (Waters 600E pump and U6K sample injector) was used to measure both biotinidase and lipoamidase using the same gradient program of Table 2.

Table 2
Elution programme for ODS column routinely used for biotinidase and lipoamidase assay[a]

Time (min)	Flow rate (ml/min)	Solvent A (%)	Solvent B (%)
0.00	1.65	100	0
2.00	1.65	100	0
2.01	3.00	0	100
6.50	3.00	0	100
6.51	3.00	100	0
10.50	3.00	100	0
10.51	1.65	100	0
12.00	1.65	100	0

[a] Waters 600E HPLC pump was used. Injector was Waters U6K with a sample-loop of 2-ml internal volume. Column temperature was at 8 °C. Solvent A, 0.1 M sodium phosphate buffer (pH 2.1): methanol = 90:10 (v/v). Solvent B, methanol. Curve type used was 1, i.e., step gradient. Other conditions were as described in Section 2.

Usually, 1 day (8 h) is sufficient to measure all the three kinetic parameters (K_m, V, and K_{ip}). After the assay, the Develosil ODS column was washed at 72 °C in the column oven with an eluent (mixture of aqueous solvent A: methanol = 40:60 (v/v)) at a flow-rate 1.65 ml/min in order to wash out the remained lipidic materials. The intraassay coefficient of variation (CV) was 0.8% ($n = 6$) when 50 pmol of AQ was used (ref. [4], p. 440); however, the average CV of the within-day repeatability was 3.1 ± 0.82% ($n = 15$ days) when 11.5 pmol of AQ was used as in the study. The day-to-day repeatability was measured for 15 days at 11.5 pmol level of AQ, and the average peak height was 127 ± 9.6 mm with CV 7.56%.

Usually, substrate concentrations used were 5, 7.5, 10, 15, and 20 μM for BAQ, and 5, 10, 15, 20, and 25 μM for LAQ. BAQ was dissolved at 20 μM in 0.1 M Tris–HCl buffer (pH 7.0; containing 10 mM 2-mercaptoethano, 1 mM EDTA, and 10% v/v glycerol; reaction buffer), and diluted with the reaction buffer. Synthesized LAQ was dissolved at 200 μM in chloroform. This stock solution was stored at −80 °C. 2.5, 5, 7.5, 10, and 12.5 μl of LAQ solution was added into the test tube (10 mm × 75 mm), and chloroform was evaporated under the stream of nitrogen. Then, 0.09 ml of the reaction buffer was added. Reaction was started by adding 0.01 ml of the enzyme solution. The reaction was allowed to proceed for 10–60 min at 37 °C, then stopped by adding 0.05 ml of methanol–acetonitrile mixture (50:50, v/v; stopping solution). The reaction was completely inhibited by this stopping solution (Table 1). After filtering the stopped mixture by Ekicrodisc 13CR, a portion (0.005–0.06 ml) was injected into the HPLC system.

Lineweaver–Burk plot was consistently used. Although Eadie–Hofstee plot was sometimes employed to confirm the result of low V samples (V less than 10 pmol/min per mg of protein) [11], Lineweaver–Burk plot was found to be satisfactory (data not shown).

A_{mo} (k_{cat}/K_m) was calculated from K_m and V by assuming Mr of glycoprotein biotinidase and lipoamidase at 66,000 [12] and 140,000 [13], respectively, and single catalytic center. K_{ip} and K_{is} were determined as follows. Biotin inhibition (competitive inhibition by biotin; K_{ip}) for BAQ hydrolysis was measured under the suitable biotin concentration of 0.1, 0.2, 0.5, or 1 mM. Substrate inhibition (competitive inhibition by other biotin-amide substrate of BPAB or biocytin; K_{is}) for BAQ hydrolysis was also measured under the similar concentration to K_m for BAQ, i.e., ca. 10 μM. Lipoic acid inhibition for LAQ hydrolysis was also measured under the suitable lipoic acid concentration of 0.2, 1.0, 2.0, or 10 mM. D-Biotin was dissolved in methanol of 1 mM (244 mg/l) and stored at 8 °C wrapped with foil of aluminium to prevent the unexpected esterification reaction. DL-α-Lipoic acid was also dissolved in methanol of 20 mM and filtered by Ekicrodisc 13 CR, and used directly or after diluting to 2.0 mM with methanol, and stored similarly as in the case of biotin. Methanol was evaporated under the stream of nitrogen gas. Standard 6-aminoquinoline (AQ; 6.64 mg) from Aldrich was dissolved in 1 L of distilled water containing 8 μl of conc. HCl. This stock standard solution (46.1 μM; stored at 4 °C wrapped with aluminium foil) was further diluted with water 20-fold. To make a calibration line a 0.005 ml of this diluted AQ standard solution was injected (11.5 pmol) into the HPLC system. Specific activity was expressed as pmol/min per mg of protein.

Since the final protein concentration in the reaction mixture must be less than 0.1 mg/ml, tissue and bacterial samples were appropriately diluted with the homogenization buffer.

K_{ip} and K_{is} were calculated from the following equation, i.e.,

$$\frac{1}{v} = \frac{1}{V} + \left\{ \frac{K_m \times (1 + [I]/K_{ip}(\text{or } K_{is}))}{V \times [S]} \right\} \quad (1)$$

Therefore,

$$K_{ip}(\text{or } K_{is}) = \frac{[I]}{((1/v - 1/V) \times V \times [S])/K_m - 1}. \quad (2)$$

In line with the definition of $A_{mo} = k_{cat}/K_m$ ($s^{-1} \times M^{-1}$), we would similarly like to define $R_{ep} = k_{cat} \times K_{ip}$ ($s^{-1} \times M \times 10^{-3}$) in this text. Then, the full capacity of the enzyme would also be defined as $C_{ap} = (A_{mo} \times R_{ep})^{1/2}$ (s^{-1}) (see Fig. 3). The kinetic constants of K_m and K_i (both of K_{ip} and K_{is}) reflect those of pure enzyme, and are independent of the protein concentration. On the contrary, V, A_{mo}, R_{ep}, and C_{ap} are called the apparent parameters because these parameters depend on the protein concentrations of homogenates, supernatant fraction, and membrane fraction. Therefore, V, A_{mo}, R_{ep}, and C_{ap} are called as specific activity, specific affinity, specific repulsion, and specific enzyme capacity, respectively. Nevertheless, these specific parameters are still important for considering the truly working enzyme in the crude samples.

2.5. Inhibition test by PCMB and PMSF

Inhibition test by using PCMB (*para*-chloromercuribenzoate) was performed without the addition of 2-mercaptoethanol as described previously [12]. Inhibition test by using PMSF (phenylmethanesufonyl fluoride) was also performed as described previously [12] in the presence of 2-mercaptoethanol.

2.6. Statistics

Non-parametric statistical analysis was performed according to ref. [14].

3. Results and discussion

3.1. Assay procedure

It was previously found that the biotinidase and alanine excretions were increased in the diabetic urine and that the early changes of biotinidase and alanine metabolisms occurred in the diabetic kidney [15]. Therefore, we intended to improve further the previously described HPLC biotinidase- and lipoamidase-assay methods [3–5], in order to measure the enzyme kinetic parameters of the tissue specimens, using a single programme (Table 2) and a single HPLC system. We found that the final protein (enzyme) concentration below 0.1 mg/ml of the reac-

tion mixture was essential for the linearity of the reaction and to obtain the Michaelis–Menten-type reaction. We recognized that measuring the initial reaction rate was also important, i.e., the reduction of substrate concentration and the increment of product concentration should be less than 10% of the initial concentration to obtain the Michaelis–Menten-type reaction.

Further, it was also found that the high-ionic concentrations such as 0.1 M neutral sodium phosphate buffer (serum dilution buffer [8,9]), 154 mM NaCl solution (saline) [6], or the presence of EDTA, reduced the brain enzyme activities. Therefore, 1 mM sodium phosphate buffer (pH 7.0, without EDTA) with 10% glycerol was used as the homogenization buffer for tissues and cells [10]. Furthermore, it was found that the freeze–thaw treatment within one or two times was desirable, since lipoamidase activity was relatively labile to this treatment. Thus, prepared homogenates, supernatant fractions, and membrane fractions were stored in the aliquots of storing tubes. Detection limit of V by this method is ca. 1.0 pmol/min per mg of protein. With respect to the variation of the kinetic parameters, we measured serum biotinidase activity of Balb/c mouse (8 weeks of age and female) for consecutive 3 days. V was 124 ± 1.41 pmol/min per mg of protein (CV = 1.14%), K_m was 12.2 ± 0.80 μM (CV = 6.59%), and A_{mo} was $13.0 \pm 1.0\ s^{-1} \times M^{-1}$. In order to assess the stability of the mouse enzyme, comparison of with and without pre-incubation and with pre-incubation at 37 °C for 3 h was performed for 3 days. The kinetic parameters V, K_m, and A_{mo} were the same between with and without pre-incubation, and this finding was reproduced for 3 days.

Since kinetic measurement is performed at relatively low substrate concentrations around K_m (5–20 μM) and with small reaction volume of 0.1 ml, kinetic parameter measurement can be carried out using a small amount of BAQ and LAQ (3.86 μg/day), i.e., 1 mg amount of BAQ or LAQ is sufficient for 260 days' assays (or for about 1 year's assays) giving the high-performance enzyme assay.

3.2. Biotinidase distribution

The result of biotinidase distribution as expressed by V (specific activity) in the rat is shown in Table 3. As a reference, previous results (velocity at 150 μM of biotinyl-4-aminobenzoate as substrate) are also incorporated. Specific activity (V) of biotinidase is highest in the LEW rat kidney (Table 3), which is in line with previous results [2,6,16]. Specific activity of serum of LEW rat is second higher, i.e., male serum is 151 pmol/min/mg of protein and female serum is 120 pmol/min/mg of protein, respectively (these data of body fluid are not shown in Table 3). Thus, V of LEW rat serum becomes 9.92 nmol/min/ml for male and 9.29 nmol/min/ml for female using the published serum protein values of 65.7 mg/ml for male and 77.4 mg/ml for female, respectively [8]. This high serum activity of LEW rat seems to be a species-dependent phenomenon, since V of mouse serum is low (see Table 8 below).

The affinity parameters of biotinidase are also summarized in Table 4. A_{mo} (specific affinity) is highest in the left cerebrum. Significant region difference between left and right cere-

Table 3
Summary of the specific activities of biotinidase in the rat[a]

Tissue	Specific activity of biotinidase (pmol/min/mg of protein)			
	Ours (by V)	Knappe et al.	Pispa	Nilsson et al.
		(assayed at 150 μM of biotinyl-4-aminobenzoate)		
Kidney[b]	196	105	134	(86)
Testis	109		33	
Ovary	95		58	
Small intestine[b]	67		37	(37)
Spleen	63		17	(35)
Liver[b]	62	36	64	70
Large intestine[c]	62 (♂, 41; ♀, 82)		33	(24)
Lung[c]	61		19	(31)
Cerebellum	56 (♂, 48; ♀, 63)			
Cerebrum	54 (♂, 43; ♀, 65)		(0) (brain)	(12) (brain)
Stomach[b]	52		18	(31)
Esophagus[b]	46			
Bone marrow[c]	25			
Abdominal skin	24			
Heart[c]	23	30	8	(16)
Pancreatic tail[c]	21		(11) (pancreas)	(19) (pancreas)
Pancreatic head[c]	15			
Thigh muscle[b]	12		2	(4)
Uterus	8		42	
Diaphragm	4			(12)

[a] Knappe et al. [2]; measured at 150 μM of biotinyl-4-aminobenzoate. Pispa [16]; SD rat (outbred) was used. Nilsson et al. [6]; only liver specific activity was presented by using Lowry's method for protein assay. Other values were estimated by us from the values of wet-weight base, and female Wistar rat (outbred) was used. Our assay condition was as described in Section 2 using LEW rat (inbred; mean of male and female).

[b] No gender difference in biotinidase.

[c] Presence of affinity differences between male and female.

Table 4
The result of biotinidase kinetics determination in the male and female LEW rat[a]

Tissue	Biotinidase (male)			Tissue	Biotinidase (female)		
	A_{mo} ($s^{-1} \times M^{-1}$)	K_m (μM)	V (pmol/min/mg)		A_{mo} ($s^{-1} \times M^{-1}$)	K_m (μM)	V (pmol/min/mg)
Cerebrum (left)	24.6	2.43	47.2	Cerebrum (left)	35.0	2.97	81.6
Testis	22.4	6.17	109	Kidney	30.3	7.97	191
Kidney	21.3	11.9	200	Large intestine	27.7	3.75	81.9
Bone marrow (duodenum)	18.1	1.71	24.5	Small intestine	25.8	2.67	54.3
Stomach (corpus)	17.9	3.36	47.5	Stomach (corpus)	25.3	3.88	77.5
Upper labial skin	15.4	0.75	9.1	Lung	23.8	3.69	69.4
Cerebellum	14.6	4.17	48.1	Ovary	22.1	5.45	95.1
Large intestine	12.3	4.27	41.3	Cerebellum	19.0	4.17	62.5
Small intestine (duodenum)	12.1	7.75	74.1	Heart 19.0	1.61	24.1	
Small intestine (jejunum)	12.1	5.81	55.4	Upper labial skin	15.4	1.18	14.3
Cerebrum (right)	11.1	4.39	38.3	Spleen	13.8	5.41	58.8
Liver	10.1	8.23	65.8	Cerebrum (right)	13.0	4.63	47.4
Spleen	9.8	8.58	66.2	Lower labial skin	12.1	1.82	17.4
Lung	8.4	7.91	52.1	Liver	10.8	6.76	57.5
Small intestine (ileum)	8.4	12.1	80.3	Bone marrow	7.6	4.15	24.8
Heart (ileum)	8.2	5.49	35.7	Small intestine	6.2	13.9	67.6
Esophagus (jejunum)	5.8	10.1	46.4	Small intestine	5.8	15.1	69.4
Lower labial skin	5.3	4.77	19.8	Esophagus	5.8	10.1	46.4
Stomach (pylori)	4.5	11.6	41.0	Pancreatic head	5.3	3.87	16.3
Diaphragm	3.5	1.87	5.1	Stomach (pylori)	4.5	11.6	41.0
Thigh muscle	3.0	4.77	11.3	Pancreatic tail	3.6	4.39	12.4
Abdominal skin	2.6	11.9	24.1	Thigh muscle	2.6	6.25	12.8
Pancreatic tail	1.8	21.7	30.3	Uterus	2.5	3.90	7.80
Pancreatic head	0.9	19.2	13.5	Diaphragm	2.3	1.87	3.40
				Abdominal skin	1.9	11.0	16.1

[a] Assay conditions were as described in Section 2 using LEW rat (8 weeks of age).

brum is also obtained as shown in Fig. 1 ($p < 0.01$, $n_1 = n_2 = 5$; Mann–Whitney's U test). However, this difference of A_{mo} turned out to be specific for LEW rat, i.e., A_{mos} of male ICR mouse were 36.7 $s^{-1} \times M^{-1}$ (right cerebrum) and 5.1 $s^{-1} \times M^{-1}$ (left cerebrum), but those of female ICR mouse were 17.3 $s^{-1} \times M^{-1}$ (right cerebrum) and 29.4 $s^{-1} \times M^{-1}$ (left cerebrum), respectively. Tissues with large A_{mo} values (such as cerebrum and kidney) may require plenty of biotin. It is important that small K_m values are found in the male bone marrow and female heart (Table 4). K_{ms} of upper labial skin and diaphragm of both male and female are also small (Table 4). Biotin may be important in these rat tissues, although specific activities (Vs) are not so high.

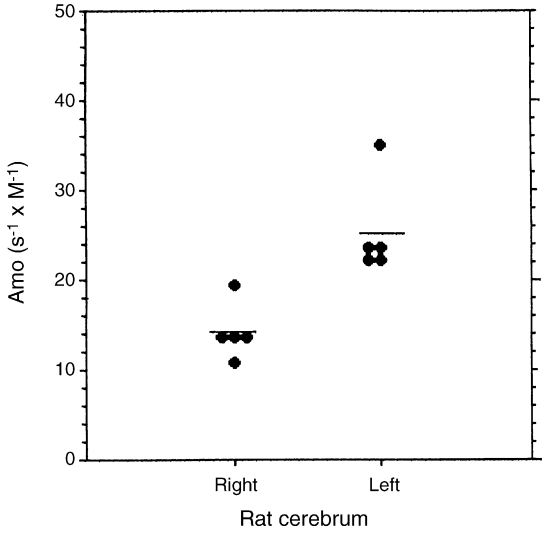

Fig. 1. Differences in the specific affinity (A_{mo}) of biotinidase of the LEW rat cerebrum between left and right regions. Cerebra of both genders ($n = 5$) were used, since sex difference was not detected in the LEW rat cerebrum. There was a significant difference ($p < 0.01$; Mann–Whitney's U test) between left region and right region in the LEW rat cerebrum. Other conditions for measurement were as described in Section 2.

In Table 5, the results of kinetic studies were summarized using several representative rat tissues and serum together with some bacteria. In *Lactobacillus casei* (*Shirota* strain), the highest V (375 pmol/min/mg of protein) of biotinidase was observed (Table 5), which is in line with the previous report of Knappe et al. [2]. However, another *Lactobacillus casei* (*NY 1301* strain) showed a lower activity (Table 5), and strain differences seemed to be present between these *Lactobacillus casei* strains.

3.3. Characteristics of thiol-type biotinidase/lipoamidase of Mr 66,000

Rat kidney biotinidase is noncompetitively inhibited by PCMB as shown in Fig. 2 (upper panel). Thus, rat kidney and human serum biotinidases are typical thiol-type enzymes [12]. By contrast, bacterial biotinidase of *Lactobacillus casei* is not a thiol-type one (Fig. 2, lower panel). We also indicated the characteristics of biotinidase in Table 5, i.e., rat serum biotinidase was allosterically influenced by lipoic acid, but bacterial *Lactobacillus casei* enzyme was competitively inhibited by lipoic acid.

Table 5
Summary of kinetic parameters in several representative specimens

Substrate	BAQ					
Specimens	A_{mo}[a]	K_m	V	K_{ip} (biotin)	R_{ep}[a]	C_{ap}[a]
Lactobacillus casei (*Shirota*)	123 (K_i by lipoic acid 55)[b]	3.85	375	2790	1320	403
Lactobacillus casei (*NY1301*)	22	5.32	91.7	1010	117	50.5
Bacillus natto	<1.0	–	<1.0	–	–	–
LEW rat serum (♂)	82	2.56	165	101	21.2	41.7
	(allosteric effect by 0.2 mM lipoic acid = 23% increase of A_{mo})					
LEW rat liver (perfused; ♀)	12	6.06	58.8	141	10.3	11.1
	(allosteric effect by 1 mM lipoic acid = 46% increase of A_{mo})					
LEW rat kidney (♂)	21	11.9	200	194	48.5	31.9
LEW rat heart (♂)	8.2	5.49	35.7	411	18.5	12.3
Substrate	LAQ					
Specimens	A_{mo}	K_m	V	K_{ip} (lipoic acid)	R_{ep}[a]	C_{ap}[a]
Lactobacillus casei (*Shirota*)	231 (K_i by biotin 543)[b]	2.25	411	564	293	260
Bacillus natto	71.3	5.88	331	ND	ND	ND
LEW rat serum (♂)	8.8	29	202	44.3	11.3	9.97
	(allosteric effect by 0.5 mM biotin = 11% increase of A_{mo})					
LEW rat liver (perfused; ♀)	259	6.45	1320	317	530	370
	(apparently independent of 0.5 mM biotin)					
LEW rat kidney (♂)	15.8	17.4	217	ND	ND	ND
LEW rat heart (♂)	2.8	29.5	64.5	ND	ND	ND

[a] Assay conditions and definitions of kinetic parameters were as described in Section 2.
[b] Membrane fraction was used. ND = not yet determined.

Bacterial *Lactobacillus casei* enzyme is a membrane enzyme (see Section 3.7), and the same enzyme and same catalytic center may be handling both biotin and lipoic acid similarly. On the other hand, purified thiol-type serum biotinidase (*M*r 66,000) of humans is also the purified thiol-type lipoamidase [20,21], and rat serum and liver enzymes seem to be similar to the human counterparts (Table 5, upper portion). Thus, this *M*r 66,000 thiol-type enzyme would suitably be called a thiol-type biotinidase/lipoamidase. This thiol-type biotinidase/lipoamidase is distributed throughout nearly all the tissues; therefore, purified human milk biotinidase [22], porcine cerebrum biotinidase [18], guinea pig liver biotinidase [23], and human urine biotinidase (our unpublished observation) are all able to hydrolyze both lipoic acid-amide substrate and biotin-amide substrate.

3.4. Kinetic parameters of lipamidase

Kinetic parameters of lipamidase were also measurable by this HPLC method, and the results were also shown in Table 5 (lower portion). Both bacteria of *Lactobacillus casei* (*Shirota*) and *Bacillus natto* exhibited lipoamidase activity. Although *Lactobacillus casei* (*Shiota*) possessed both biotinidase and lipoamidase activities, *Bacillus natto* possessed only the lipoamidase activity. Lipoamidase activity of rat serum showed allosteric effect by biotin. The reason of this is because lipoamidase activity of serum is due mostly to thiol-type biotinidase/lipoamidase, and esterase-type lipoamidase is very few in the serum. In contrast to *Lactobacillus casei* (*Shirota*) enzyme, thiol-type biotinidase/lipoamidase of rat serum seems to have two mutually influencing active centers. Rat liver lipoamidase activity is due mostly to the esterase-type lipoamidase (see Section 3.6); therefore, the effect of biotin is not apparently detectable in the rat liver (Table 5, lower portion).

3.5. Significance of specific affinity A_{mo} (k_{cat}/K_m) and K_{ip}

Using the membrane fraction of *Lactobacillus casei* (*Shirota*), we found that k_{cat}/K_m value was decreased to about 10-fold by the urea treatment, whereas increases of both parameters K_m and V were observed (data not shown). Therefore, we recognized that k_{cat}/K_m was a more suitable affinity parameter than K_m, and we designated this parameter as A_{mo}. Significance of the constant K_{ip} (by a hydrolytic product of biotin) as a good indicator for the product repulsing force was also recognized, and the typical results were also shown in Table 5. The K_{ip} values by biotin are largest in the *Lactobacillus casei* (*Shirota* and

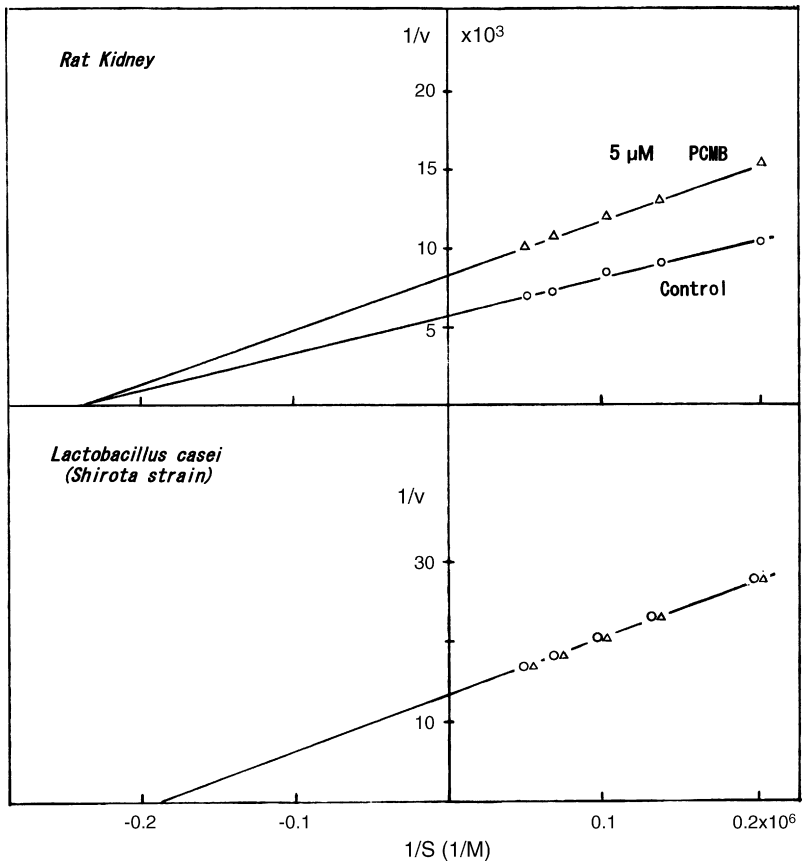

Fig. 2. Effect of PCMB (*para*-chloromercuribenzoate) on biotinidase activity at various substrate (BAQ) concentrations. Upper panel: rat kidney homogenate. Open circle: control, open triangle: with 5 μM PCMB. PCMB showed a typical noncompetitive inhibition. Lower panel: *Lactobacillus casei* (*Shirota*) homogenate. Open circle: control, open triangle: with 5 μM PCMB. PCMB (at 5 μM) showed no effect on the *Lactobacillus casei* (*Shirota*) biotinidase. Final protein concentration in the reaction mixture was 0.01 mg/ml. In these tests, 2-mercaptoethanol was not added to the reaction mixture. Other conditions for measurement were as described in Section 2.

NY1301) and in the rat heart (Table 5). Large K_{ip} value (780 μM) for biotinidase of *Lactobacillus casei* has already been reported by Knappe et al. [2]. They first recognized that this K_{ip} was the dissociation constant of enzyme-biotin complex [2]. Therefore, we also consider that this large K_{ip} value is the resistance to the product inhibition (or repulsion force against the product of biotin). Michaelis and Menten originally considered that the affinity constant should be expressed as [enzyme-substrate complex]/[enzyme] × [substrate] (at p. 363 in ref. [1]), i.e, affinity constant is the reciprocal of K_m. Similarly, we would like to suggest that the repulsion (R_{ep}) is [enzyme] × [product]/[enzyme-product complex]. Enzyme should have both high affinity for the substrate and high repulsion against the product in order to execute the high enzymatic capacity (C_{ap}) during substrate handling (Fig. 3). As in the case of definition of A_{mo}, we would like to define $R_{ep} = k_{cat} \times K_{ip}$ ($s^{-1} \times M \times 10^{-3}$) and $C_{ap} = (A_{mo} \times R_{ep})^{1/2}$ (s^{-1}). Therefore, highest biotinidase and lipoamidase affinity and capacity resided in *Lactobacillus casei* (*Shirota*) and in rat liver, respectively (Table 5).

Competitive inhibition by the other substrate (K_i by another substrate; K_{is}) than BAQ substrate has also been tested by using normal human liver. The K_{is} by biocytin was 5.54 μM and that by BPAB was 7.25 μM, respectively. The K_m for BAQ of this liver was 7.25 μM. Therefore, competitive inhibition by the similar substrates was performed at the similar concentrations to K_m. Since, K_{is} is the typical constant of competitive inhibition to compete the active center with BAQ substrate. Therefore, the value of K_{is} becomes similar to the value of the K_m of BAQ and is considered to be related to the enzyme affinity to the various substrates. Thus, K_{is} is the reflection of the

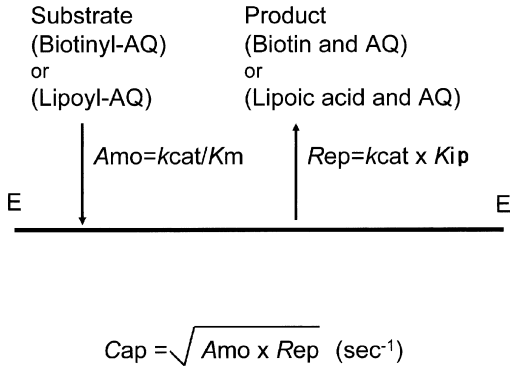

Fig. 3. A model by the Cleland's notation for substrate- and product-handling by the hydrolase enzyme of biotinidase or lipoamidase. Total enzymatic capacity is expressed as the root of the product of the substrate handling (A_{mo}) and the enzymatic product handling (R_{ep}), i.e., by the C_{ap} (numbers of the substrate molecules handled per second). Other details were as described in Section 2.

enzyme affinity to the substrate, i.e., biocytin may have the highest affinity to the enzyme biotinidase among these three substrates.

Furthermore, the determination of K_{ip} turned out to be an important issue in the research of the human diseases, i.e., the increase of the liver K_{ip} in biotinidase at the cancer tissue as compared to the surrounding non-cancer normal tissue was found in the human livers with hepatocellular carcinoma (manuscript in preparation).

3.6. Characteristics of PMSF-inhibited esterase-type lipoamidase of Mr 140,000

LAQ substrate is hydrolyzed by both of the thiol-type biotinidase/lipoamidase (*M*r 66,000) and by the PMSF-inhibited esterase-type lipoamidase (*M*r 140,000). Thus, we tested PMSF for the liver lipoamidase activity, and found that more than 99% of liver lipoamidase activity was due to PMSF-inhibited esterase-type lipoamidase. Residual lipoamidase activity after PMSF-inhibition was completely inhibited by the further addition of PCMB. This residual lipoamidase activity was due to the thiol-type biotinidase/lipoamidase. Therefore, PMSF-inhibited esterase-type lipoamidase was estimated by subtracting the biotinidase activity from the total lipoamidase activity, and the results are summarized in Table 6. K_{ms} and A_{mos} for LAQ are also shown in Table 6. K_{ms} of esterase-type lipoamidase are larger than those of thiol-type biotinidase/lipoamidase; however, those of cerebellum of both female and male (8.8 μM) and female liver (6.5 μM) were small. Lipoic acid may play a special role in the cerebellum [24].

PMSF-inhibited esterase-type lipoamidases were already purified by us. Human milk lipoamidase [25] has turned out to have the same amino acid sequence to cholesterol esterase (bile salt-stimulated lipase) [26]. Purified porcine brain lipoamidase is very similar to acetylcholinesterase [19,27], and brain enzyme shows more complex characteristics in the active center [17]. These PMSF-inhibited esterase-type lipoamidases all resided in the membrane fraction, except secreted-enzyme of human milk lipoamidase.

3.7. Subcellular distributions

The result of subcellular distribution of biotinidase is summarized in Table 7. Biotinidase/lipoamidase of *Lactobacillus casei* (*Shirota*) resided in the membrane fraction, i.e., A_{mos} of lipoamidase in homogenate, membrane-, and supernatant-fraction were 231, 241, and 57.3 $s^{-1} \times M^{-1}$, respectively. However, lipoamidase activity of *Bacillus natto* resided mainly in the supernatant fraction (data not shown). In the LEW rat, the thiol-type biotinidase/lipoamidase of gonads resided in the supernatant fraction, but enzyme of kidney and liver resided in the membrane- and in the supernatant-fractions equally (Table 7). Female rat heart and male rat liver enzymes of the membrane fraction showed smaller K_{ms} than those of the supernatant fraction

Table 6
Specific activity (V), K_m, and A_{mo} values of PMSF-inhibited esterase-type lipoamidase in the LEW rat[a]

Tissue	PMSF-inhibited lipoamidase					
	Male			Female		
	V (pmol/min/mg)	K_m (μM)	A_{mo} ($s^{-1} \times M^{-1}$)	V (pmol/min/mg)	K_m (μM)	A_{mo} ($s^{-1} \times M^{-1}$)
Liver[b]	1890	21.5	54.1	1260	6.5	119
Small intestine (duodenum)[b]	1160	55.6	12.8	670	49.8	8.3
Small intestine (jejunum)	739	47.2	9.6	725	47.1	9.5
Small intestine (ileum)[b]	746	109	4.2	312	109	1.8
Pancreatic head	699	22.0	19.5	502	18.9	16.3
Pancreatic tail	217	41.5	3.2	239	39.2	3.8
Cerebrum[b]	213	18.5	7.1	364	17.6	10.8
Cerebellum[b]	260	8.8	18.2	154	8.8	10.8
Large intestine[b]	204	54.1	2.3	0[c]		
Testis	173	15.5	–			
Stomach (pylori)	111	93	0.73	80	80.0	0.62
Stomach (corpus)	36	22.2	1.0	130	26.3	3.0
Esophagus	79	87	0.56	79	87	0.56
Lung[b]	74	33.8	1.4	0[c]		
Spleen	57	35.7	0.98	58	23.8	1.5
Heart[b]	29	29.5	0.61	123	37.3	2.0
Kidney[b]	17	17.4	0.60	79	22.2	2.2
Diaphragm	14	60.2	0.14	7	25.3	0.17
Uterus	–			32	66.7	0.30
Ovary[c]	–			0		
Bone marrow[c]	0			0		
Abdominal skin[c]	0			0		
Thigh muscle[c]	0			0		

[a] Lipoamidase activity (V) minus biotinidase activity (V of Table 4).
[b] Presence of sex differences between male and female.
[c] Only the thiol-type biotinidase/lipoamidase activity is present in these tissues.

Table 7
Subcellular distribution between membrane- and supernatant-fractions[a]

Bacterial cells and rodent tissues	Biotinidase		
	V (pmol/min/mg)	K_m (μM)	A_{mo} ($s^{-1} \times M^{-1}$)
Lactobacillus casei (*Shirota strain*)			
Homogenate	375	3.85	123
Membrane	1050	5.56	239
Supernatant	207	3.83	68.5
Mouse kidney (ICR strain) (♀)			
Homogenate	385	15.5	31.4
Membrane	191	16.7	14.5
Supernatant	101	16.7	7.6
LEW rat kidney (♂)			
Homogenate	200	11.9	21.3
Membrane	250	12.5	25.3
Supernatant	238	12.5	24.3
LEW rat liver (♂)			
Homogenate	65.8	8.23	10.1
Membrane	33.3	8.23	5.13
Supernatant	101	15.4	8.28
LEW rat ovary (♀)			
Homogenate	95.1	5.45	22.1
Membrane		Not detectable	
Supernatant	89.3	5.71	19.8
LEW rat testis (♂)			
Homogenate	109	6.17	22.4
Membrane	23.3	6.02	4.9
Supernatant	60.2	6.02	12.7
LEW rat heart (♀)			
Homogenate	21.2	1.67	16.1
Membrane	3.5	1.11	4.0
Supernatant	35.7	7.75	5.8

[a] Ultracentrifugation was performed in the homogenization buffer at 100,000 × *g*, 90 min, at 4 °C. Other conditions were as described in Section 2.

(Table 7); therefore, membrane enzyme may be more important in these tissues.

3.8. Species differences

As shown in Table 8, thiol-type biotinidase/lipoamidase of the LEW rat serum showed the highest A_{mo} value than the other tissues. On the other hand, A_{mo} of C57BL/6 mouse serum biotinidase is lower than the other tissues (Table 8). This is mainly due to the differences in K_m, i.e., K_{ms} of human serum [4], LEW rat serum, and C57BL/6 mouse serum are 10, 2.5, and 22 μM, respectively. The reason of these species difference is not clearly understood; however, chemical structures of the enzymes among these three species may be different.

3.9. Tissue regional differences

In Table 9, typical regional differences of thiol-type biotinidase/lipoamidase within several tissues and bloods were summarized. Skin, stomach, gut, and cerebrum showed tissue region differences of specific affinity (A_{mo}), and also pancreas showed the affinity difference with respect to the esterase-type lipoamidase (Table 9). Therefore, precautions would be necessary when studying these tissues.

Table 8
Typical species difference in the thiol-type biotinidase/lipoamidase of the major tissues between male LEW rat and male C57BL/6 mouse[a]

Tissue	Thiol-type biotinidase/lipoamidase		
	A_{mo} ($s^{-1} \times M^{-1}$)	K_m (μM)	V (pmol/min/mg of protein)
Male LEW rat			
Serum	77.7	2.47	151
Testis	22.4	6.17	109
Kidney	21.3	11.9	200
Small intestine (jejunum)	12.1	5.81	55.4
Liver	10.1	8.23	65.8
Stomach (pylori)	4.5	11.6	41.0
Male C57BL/6 mouse			
Liver	9.3	12.8	94.3
Small intestine (jejunum)	7.1	9.80	54.6
Kidney	6.7	10.4	54.9
Testis	1.9	20.8	31.3
Stomach (pylori)	1.8	29.0	41.3
Serum	0.89	21.7	15.2

[a] Assay conditions using BAQ as substrate were as described in Section 2.

3.10. Individual and age differences in the case of human serum

Specific activities of human serum thiol-type biotinidase/lipoamidase (at substrate BAQ concentration of 40 μM) were measured on 110 persons of various ages and diseases. The result is shown in Fig. 4. On the contrary to the inbred LEW rat, humans are not genetically inbred at all, and the individual difference in human serum biotinidase activity is extreme (Fig. 4). Therefore, we could successfully accumulate the enzyme kinetic parameters and constants systematically (as shown in this text) only by using the LEW rat. Although there was such a large individual difference in the human serum, it was found that the age difference is observable as shown in Fig. 4. A quadratic regression curve (or regression non-linear line) is found, i.e., the highest

Table 9
Affinity (A_{mo}) differences in thiol-type biotinidase/lipoamidase between the regions of the LEW rat tissues[a]

Regions in the tissue	Differences in A_{mo}
Arterial blood and venous blood	No
Kidney (left and right)	No
Skin or Cutis (epidermis and derm (corium))	No
Abdominal skin and labial skin (cutis)	Yes (labial > abdominal)
Upper labial skin and lower labial skin	Yes (upper > lower)
Stomach (corpus and pyloric portion)	Yes (corpus > pyloric)
Cerebrum (left and right)[b]	Yes (left > right)

[a] Affinity differences in PMSF-inhibited esterase-type lipoamidase were observed in pancreatic head and tail (head > tail), in small intestine (duodenum and jejunum > ileum), and in stomach of female rat (corpus > pyloric) (see Table 6).

[b] Shown as a figure (Fig. 1), i.e., significant ($p < 0.01$; Mann–Whitney's U test).

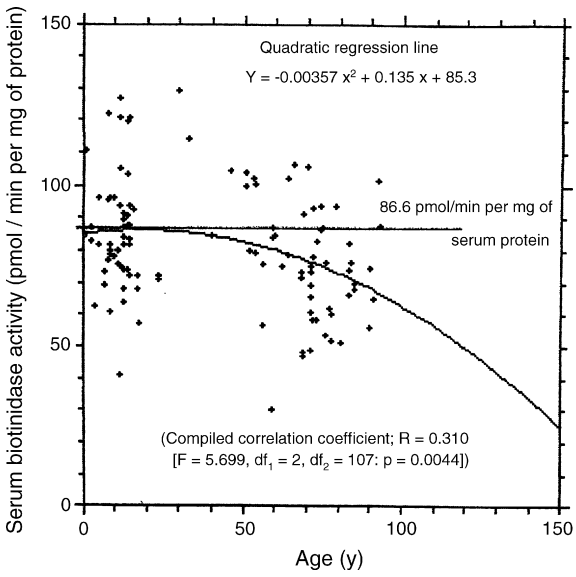

Fig. 4. Individual and age differences found in the human serum. One hundred ten human sera were measured at the BAQ concentration of 40 μM. Assay method was as described in Section 2.

activity (86.6 pmol/min/mg of serum protein) is obtainable at the human age of 18.7 years, and the activity decreases gradually with age (Fig. 4). This regression curve extrapolates to 0 of activity at the age of 174 years, which may suggest the destined length of the human life.

3.11. Conclusions

(1) This improved and reliable HPLC-fluorimetric determination method for the enzyme kinetic parameters by combining with sensitive SEC-protein assay method is shown to be successfully applicable to bacterial cells, tissues, and serum samples.
(2) Since this HPLC method is simple, automated high-throughput enzyme assay machine with high output (such as V, K_m, K_i (both K_{is} and K_{ip}), A_{mo}, R_{ep}, C_{ap}) is expected to be easily obtainable.
(3) Therefore, this method is expected to be a strong and useful tool in the analysis of the tissues and serum from those patients who suffered from many serious diseases, if it is suitably applied under the precautions, as described in this text.

Acknowledgement

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