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High-performance liquid chromatographic determination of lipoamidase (lipoyl-X hydrolase) activity with a novel substrate, lipoyl-6-aminoquinoline

Kazuyuki Yoshikawa[a,b], Kou Hayakawa[a,*], Noriyuki Katsumata[a], Toshiaki Tanaka[a], Teiji Kimura[b], Kunio Yamauchi[b]

[a]*National Children's Medical Research Center, Division of Endocrine and Metabolism, 3-35-31 Taishido, Setagaya-ku, Tokyo 154, Japan*

[b]*Department of Food Technology, College of Agriculture and Veterinary Medicine, Nihon University, 3-34-1 Shimouma, Setagaya-ku, Tokyo 154, Japan*

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Abstract

An HPLC lipoamidase (lipoyl-X hydrolase) assay method has been developed, which uses a novel fluorescent substrate, lipoyl-6-aminoquinoline (LAQ). LAQ is synthesized from lipoic acid and 6-aminoquinoline (AQ) through lipoyl chloride as an intermediate and is conveniently purified by washing with chloroform–methanol. Mechanistic studies on the time-course, the dependence on enzyme and substrate concentrations were performed by using LAQ and a model enzyme (milk lipoamidase). Moreover, this method was successfully applied to the direct determination of the lipoamidase (LAQ hydrolase) activity in samples of human liver, milk, stools and porcine serum. Using this novel synthetic lipoyl substrate, we demonstrated that LAQ hydrolase was present in some specific tissues; LAQ hydrolase was solely present in the grey matter and not in the white matter in the human cerebrum. Furthermore, LAQ hydrolase activity was shown to increase in human liver cancer. Thus, this enzyme assay method is expected to be applicable to the tissue distribution study and also to the basic research on human diseases such as cancer.

Keywords: Lipoamidase; Lipoyl-X hydrolase; Enzymes; Lipoyl-6-aminoquinoline

1. Introduction

Lipoamidase (EC number not yet given) is an amidase which hydrolyzes lipoyl-lysine to produce lipoic acid and lysine [1,2]. The enzyme also hydrolyzes some artificially synthesized lipoic acid derivatives, such as lipoyl 4-amidobenzoate (LPAB) [3]. Interestingly, lipoamidase (LPAB hydrolase) activity in human serum showed strong correlation ($r=0.8931$, $P<0.01$) to biotinidase activity [4]. Also, isolated human serum lipoamidase (M_r 76 000) had biotinidase (EC 3.5.1.12) activity [2,5–7]. Human milk lipoamidase (LPAB hydrolase; M_r 135 000), which hydrolyzes lipoyl-lysine or biocytin very slowly and has a serine-type active center [8,9], has been genetically identified as a human milk bile salt-stimulated lipase (pancreatic cholesterol esterase) [10]. Human milk biotinidase (M_r 68 000),

*Corresponding author.

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which hydrolyzes both biotinyl-4-aminobenzoate (BPAB) and LPAB (manuscript in preparation) and has a thiol-type active center [11], has different characteristics compared to the serum enzyme, with respect to molecular mass, amino acid and sugar composition.

Biotinidase (M_r 70 000) and lipoamidase (LPAB hydrolase; M_r 60 000) were isolated from guinea-pig livers [12]. Study of rat liver homogenates suggested that LPAB hydrolase and biotinidase (BPAB, biocytin and lipoyl-lysine hydrolyzing) were present [13]. Lipoyl-X hydrolase (LPAB hydrolase; M_r 40 000) [14] and biotinidase (M_r 68 000) [15] were isolated from pig brain and cerebrum, respectively. This pig-brain lipoamidase (lipoyl-X hydrolase) is able to hydrolyze lipoyl-lysine, and is a multiple hydrolase [16] containing a complex active center of serine-, thiol- and metallo-groups [14,16,17]. Pig-brain lipoamidase (LPAB hydrolase) has distinctly different substrate specificity from rat liver LPAB hydrolase, which is considered to be unable to hydrolyze lipoyl-lysine. This may be due to species difference (pig and rat) or tissue difference (liver and brain). Therefore, lipoamidase(s) from different tissues and body fluids in a single species (man) may exhibit different enzyme characteristics. In order to characterize the lipoamidase(s) from different organs or body fluids from humans, strict studies on substrate specificity must be required. However, only a limited number of substrates for lipoamidase is available; i.e., pyruvate dehydrogenase (PDH), lipoamide, lipoyl-lysine and LPAB. PDH purification requires the bacterial growth and enzyme purification procedures. Furthermore, we found that both human serum lipoamidase and pig-brain lipoamidase are not able to liberate lipoic acid from PDH [2]. The production of lipoic acid from lipoamide and lipoyl-lysine is measurable (submitted for publication); however, measurement of lipoic acid requires pre-column derivatization. Preparation of lipoyl-lysine requires repeated fractionation by RP-HPLC [18]. The substrate, LPAB, is relatively easy to prepare. However, measurement of *para*-aminobenzoic acid (PAB) may be affected by interferences of many compounds in the sample. Biotinidase assay with biotinyl-6-aminoquinoline (BAQ) was superior to biotinyl-4-aminobenzoate (BPAB), since there were less interfering peaks in the 6-aminoquinoline (AQ) assay than in the PAB assay [19].

Therefore, we have devised a new substrate, lipoyl-6-aminoquinoline (LAQ), for lipoamidase, which is convenient to prepare. Applications of this substrate to several human tissues strongly suggested the presence of tissue differences. Furthermore, LAQ was expected to be applicable to human diseases such as cancer.

2. Experimental

2.1. Chemicals and reagents

Biotin 6-amidoquinoline (BAQ) and biotin 4-amidobenzoic acid sodium salt (BPAB) were purchased from Sigma (St. Louis, MO, USA). 6-Aminoquinoline (AQ) was obtained from Aldrich (Milwaukee, WI, USA). DL-α-Lipoic acid (thioctic acid) and thionyl chloride were from Nacalai Tesque (Kyoto, Japan). Chloroform, methanol and other chemicals were highly pure substances provided by Wako (Osaka, Japan). LPAB hydrolase (milk lipoamidase or milk bile salt-stimulated lipase; BSSL) was purified as reported [8].

2.2. Lipoyl-6-aminoquinoline (LAQ) synthesis

LAQ was synthesized according to the method for LPAB described previously [4]. Briefly, DL-lipoic acid (5 mmol, 1.0 g) was dissolved in 5 ml of hot methanol and 50 ml of 0.1 *M* aqueous NaOH was added. The mixture was frozen and lyophilized to yield sodium D,L-lipoate (1.05 g). A solution of 3.75 ml thionyl chloride in 15 ml of benzene was cooled in an ice bath and stirred while 1.05 g of sodium D,L-lipoate was added in four equal portions at 15-min intervals. The reaction mixture was stirred for 2.5 h and concentrated in vacuo. The brownish residue (D,L-lipoyl chloride) was added to 50 ml of 1,4-dioxane. To this solution was added 10 ml of 1,4-dioxane dissolved in 5 mmol (0.70 g) of AQ. After 30 min at room temperature, the reaction mixture was frozen and lyophilized. The residue was triturated with warm solvent (chloroform–methanol (1:1, v/v); 100 ml, 50°C) and 50 ml of 0.1 *M* HCl was added. The mixture was centrifuged at 1500 *g* for 15 min, and the lower chloroform phase was

collected. The excess AQ was further washed four times as described by Bligh and Dyer [20], except we used 0.1 *M* aqueous HCl instead of distilled water. The washed chloroform solution was then dried over anhydrous magnesium sulfate and stored at −80°C under nitrogen gas. The amount of product obtained was 25 mg, and the product had a melting point of 172–175°C.

2.3. Specimens

Human sera were obtained from the National Children's Hospital (Setagaya-ku, Tokyo, Japan). Tissue specimens from human cerebrum (1 year old), and from human liver and intestine were kindly donated by Dr. K. Soejima from the Showa University School of Medicine (Tokyo, Japan) and Prof. T. Nagamine from the Gunma University School of Medicine (Maebashi, Gunma, Japan). Human milk samples were donated by the Tokyo Boshi-hoken-in Hospital (Setagaya-ku, Tokyo, Japan). Porcine liver, kidney and serum were purchased from Pel-Freez Biologicals (Rogers, AR, USA). Human stools was from a healthy volunteer from this institute. The specimens were stored at −80°C.

2.4. Instruments

A Waters Model 600 E HPLC (Waters Associates, Milford, MA, USA) with a gradient elution unit was used. The column was a 50×4.6 mm I.D. stainless steel tube packed manually with spherical, 10 μm silica gel particles chemically bonded with octadecylsilane (ODS) (Develosil ODS, Nomura Chemical, Japan). Sample injection unit used was a Model U6K diaphragm-type injector (Waters) with a 2-ml sample-loading loop. A line filter and a mini-guard column (10×4 mm I.D., packed with Develosil ODS gel; GL Sciences, Tokyo, Japan) were inserted between the injector and the column. Detection was carried out with a fluorimeter (F-3000, Hitachi, Tokyo, Japan) using flow-through cell (cell volume 18 μl). AQ was assayed essentially as previously described [19], except that as solvent A a 0.1 *M* sodium phosphate buffer (pH 2.1) was used instead of a 0.1% aqueous TFA solution, and that improved gradient programmes were employed (Table 1).

2.5. Lipoamidase (lipoyl-X hydrolase) assay

The enzyme assay procedure was essentially as described previously [3,4]. Synthesized LPAB (10

Table 1
Elution programmes routinely used for LAQ and LPAB hydrolase activities.

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)	Curve type
Programme 1: for LAQ hydrolase activity (AQ assay)				
0.0	3.00	100	0	*
4.0	3.00	0	100	11
5.00	3.00	0	100	11
5.01	3.00	100	0	1
10.0	3.00	100	0	1
Programme 2: for LPAB hydrolase activity (PAB assay)				
0.0	1.50	100	0	*
2.5	1.50	100	0	11
2.51	3.00	0	100	6
5.50	3.00	0	100	1
5.51	3.00	100	0	1
9.50	3.00	100	0	1
9.51	1.50	100	0	1
12.0	1.50	100	0	1

Proportioning valves A and B were used. Curve types in numbers were according to the description of the manufacturer; i.e., 1 and 11 were step gradient and 6 was linear gradient, respectively. Solvent A: 0.1 *M* aqueous sodium phosphate buffer (pH 2.1). Solvent B: methanol.

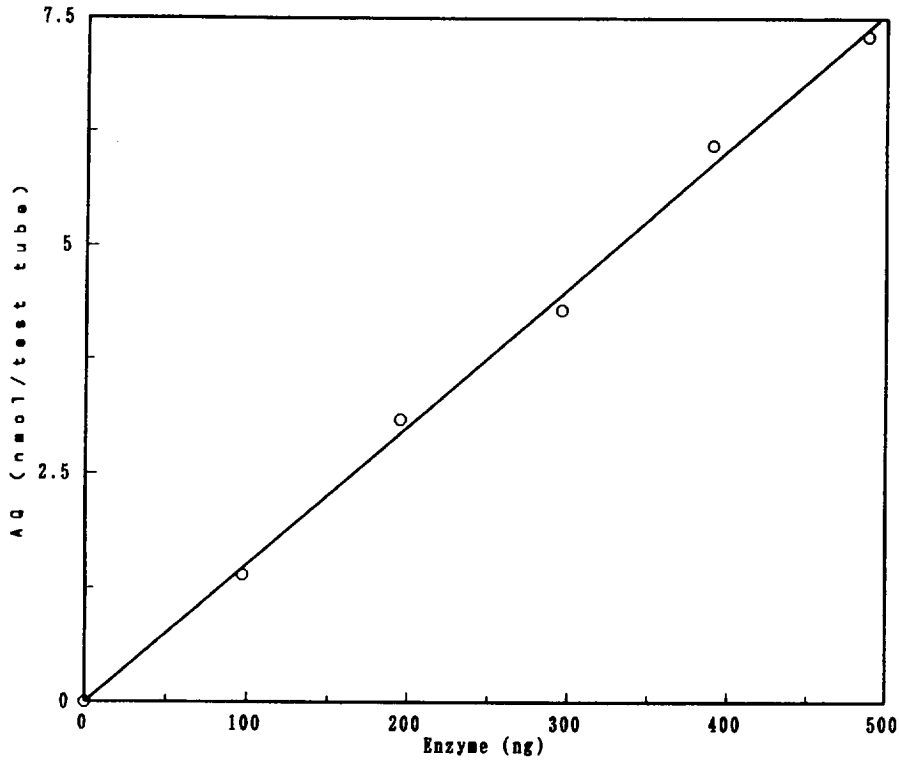

Fig. 1. Dependence of the lipoamidase reaction on the amount of enzyme. Various amounts (0.0–488 ng) of purified human milk lipoamidase (LPAB hydrolase or BSSL; 4.88 mg/ml) were incubated in the reaction mixture (0.1 ml) for 20 min at 37°C. Released AQ was measured as described in Section 2.

nmol) or LAQ (50 nmol) was dispersed in 0.09 ml of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 1 m*M* (452 mg/liter) EDTA-Na_4 salt and 10 m*M* (781 mg/liter) 2-mercaptoethanol. The substrate in reaction buffer was mixed with 0.01 ml of enzyme solution. The reaction proceeded for the appropriate time interval at 37°C, and was stopped by adding 0.1 ml of acetonitrile; i.e., the reaction mixture was diluted two-fold with acetonitrile in order to precipitate the enzyme proteins. After centrifugation and deproteinization, a portion (0.005 ml) of the clear supernatant (0.2 ml) was injected onto the HPLC system. Production of AQ was measured at an excitation wavelength of 350 nm and an emission wavelength of 550 nm [19].

2.5.1. Biotinidase (BAQ hydrolase) activity

Biotinidase (BAQ hydrolase) activity was determined as described previously using BAQ as substrate [19].

2.6. Protein content

Protein content was determined with the BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

3. Results and discussion

HPLC lipoamidase assay using LPAB as substrate has been constantly used in this laboratory since 1987 [3]. The method excludes interferences due to turbidity and has been successfully applied to human milk [8] and pig brain [14]. However, since the wavelengths we used for the detection of PAB were similar to those used for the detection of various nucleotides, the occurrence of interference remained likely. Therefore, we have recently developed an improved biotinidase assay using biotinyl-6-amino-

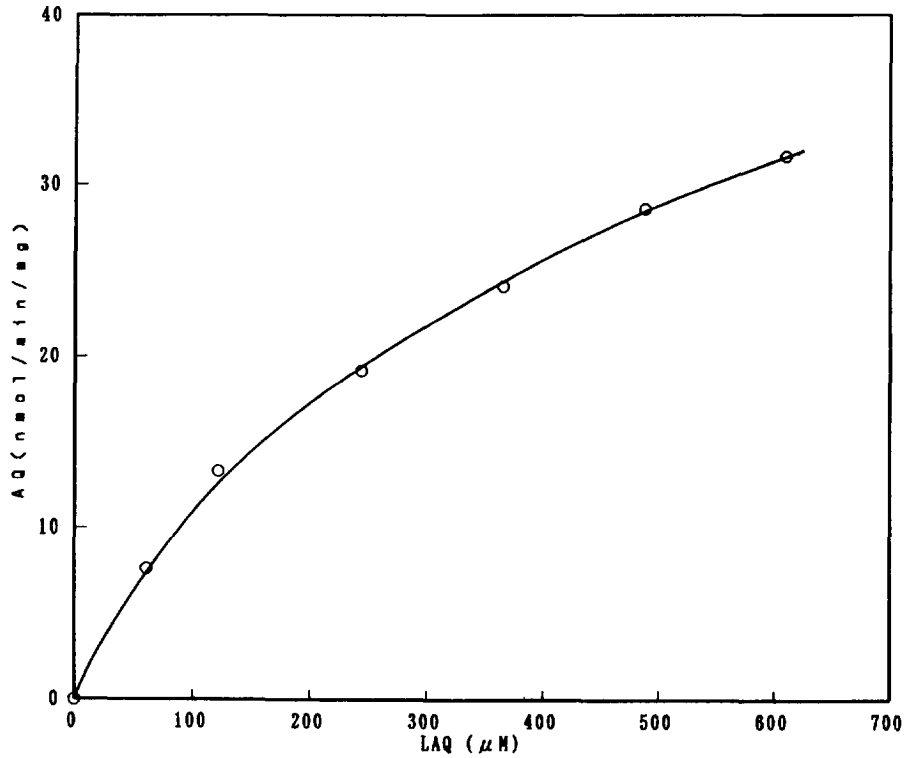

Fig. 2. Dependence of the lipoamidase reaction on the concentrations of substrate LAQ. Various amounts of LAQ (0.0–61 nmol per 0.1 ml reaction solution) were incubated with 23 μg of purified human milk lipoamidase (LPAB hydrolase or BSSL). Other conditions were as described in Section 2.

quinoline (BAQ) as substrate instead of biotinyl-4-aminobenzoate (BPAB) [19].

Accordingly, we planned to synthesize the novel lipoamidase (lipoyl-X hydrolase) substrate of lipoyl-6-aminoquinoline (LAQ). Sodium lipoate was converted to lipoyl chloride with thionyl chloride. Then, lipoyl chloride was reacted with 6-aminoquinoline to yield lipoyl-6-aminoquinoline (LAQ). The remaining AQ was completely washed out by using a mixture of chloroform–methanol–0.1 *M* aqueous HCl (2:1:1, v/v). The lower chloroform phase was collected; 25 mg of LAQ was obtained, and the melting point was 172–175°C.

Synthesized LAQ is dissolved in the neutral phosphate buffer and is tested whether it is useful for lipoamidase (lipoyl-X hydrolase) substrate or not by using milk lipoamidase as a model enzyme. The AQ assay was performed by the HPLC-fluorimetric method as described in Section 2 and gradient programme 1 (Table 1). AQ was measurable within 12 min. The detection level for AQ was similar to the previously reported result [19]. The release of AQ from LAQ by milk enzyme was linear until 60 min; boiled milk enzyme could not cause any release of AQ from LAQ at all (data not shown). The release of AQ was linearly dependent on the amount of enzyme between 0 and 500 ng (Fig. 1).

Using 23 μg of the model enzyme, the dependence of the hydrolytic velocity (specific activity) on the concentration of LAQ in the reaction buffer is shown in Fig. 2. Although the velocity curve of LAQ hydrolysis by the enzyme seemed to have a plateau, the hydrolytic velocity at concentrations higher than 700 μ*M* was not reproducible. This may be due to the limited solubility of LAQ in aqueous phosphate buffer. If the velocity curve in Fig. 2 is approximated by a hyperbola, the K_m value (concentration at the focal point) for LAQ was calculated to be ca. 120 μ*M*. The K_m value for LPAB with milk lipoamidase has been reported to be 25 μ*M* [8]. Although LAQ is

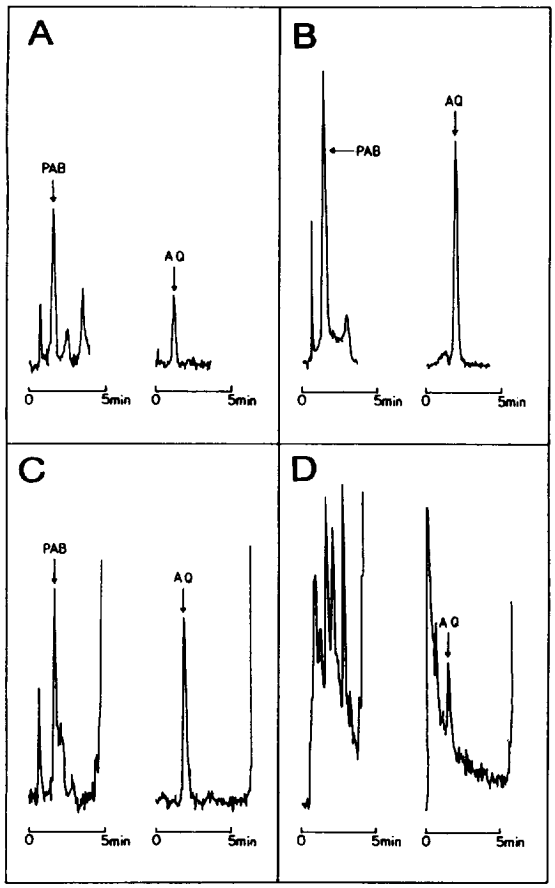

Fig. 3. Typical examples of the determination for application onto various specimens using LAQ as substrate as compared to the assay using LPAB as substrate. (A) Human liver homogenate (cancer); (B) human milk (de-fatted); (C) porcine serum; (D) human stools. Left and right chromatograms show analytical results obtained using LPAB and LAQ, respectively, as substrates. Conditions for analysis were as described in Section 2.

not so specific to milk LPAB hydrolase enzyme as compared to LPAB, LAQ is shown to be applicable to mechanistic study for purified enzyme (lipoamidase).

Since almost the highest reaction velocity was always available at the concentration of 50 nmol LAQ per 0.1 ml of reaction solution (Fig. 2), we used this concentration in the following study. As shown in Fig. 3, the results of LAQ as substrate (right chromatograms) were always superior to the results of LPAB (left chromatograms), since fewer interfering peaks were observed in the case of LAQ

Table 2
Determination of LAQ hydrolase activity as compared to LPAB and BAQ hydrolase activity in several specimens

Specimen	Activity (pmol/min per mg of protein)		
	LAQ	LPAB	BAQ
Human cerebrum			
Gray matter	27.1	19.6	3.0
White matter	ND[a]	ND	2.0
Human liver			
Hepatoma	373.3	71.0	21.2
Liver cirrhosis	91.0	65.5	34.1
Human intestine	27.9	93.5	48.6
Human serum	2.5	32.4	74.1
Human milk	972.5	51.3	2.8
Human stools	2.5	8.4	ND
Porcine liver	979.2	65.2	11.2
Porcine kidney	3418.7	775.9	32.3
Porcine serum	10.1	45.4	15.3

[a]ND=not detectable.

as substrate. The results of Fig. 3 indicate that lipoamidase assays in such samples as human liver, human milk, porcine serum and human stools are possible.

The results of tissue distribution study using LAQ as substrate with humans and porcine specimens are summarized in Table 2. As a reference the results of biotinidase (BAQ hydrolase) and lipoamidase (LPAB hydrolase) activities are also shown in Table 2. Interestingly, lipoamidase activities measured with LPAB and LAQ as substrates were detectable solely in the grey matter of human cerebrum. On the contrary biotinidase activity was detected in both grey and white matters. Furthermore, lipoamidase (LAQ hydrolase) activity in the cancer liver of humans was four-fold higher than in non-cancer liver (Table 2). Human serum showed relatively low LAQ hydrolase activity (Table 2). This may suggest that another kind of LAQ-specific lipoamidase(s) is present in human tissues than in human serum.

Porcine kidney showed the highest LAQ hydrolase activity, and porcine liver showed ca. 10-fold higher LAQ hydrolase activity than human liver (Table 2). These results suggest that the differences among species are usually observable, and that careful interpretation of the biochemical data is necessary.

We demonstrated that LAQ was applicable to the HPLC-lipoamidase assay in such turbid specimens as human tissue homogenates, human milk and porcine

serum without pretreatment. Furthermore, LAQ was expected to be applicable to studies on tissue distribution and also in human diseases.

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