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Note

Determination of lipoamidase activity by liquid chromatography with fluorimetric detection

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In a previous paper [1], we described a method for the determination of biotinidase (biotinyl-4-aminobenzoate hydrolase, EC 3.5.1.12) activity by high-performance liquid chromatography (HPLC) with fluorimetric detection. The method afforded a simple and stoichiometric assay for biotinidase by simultaneously measuring the product (*p*-aminobenzoic acid, PAB) and the residual substrate (biotinyl-4-aminobenzoate).

In the present work, we applied this stoichiometrical method to the determination of lipoamidase activity in human serum. Lipoamidase hydrolyses lipoyllysine and some artificially synthesized compounds, such as lipoamide [2]. We used lipoyl-4-aminobenzoate (LPAB) as a substrate. The HPLC method was found to be applicable to the determination of LPAB hydrolase activity (lipoamidase activity).

EXPERIMENTAL

Chemicals and reagents

D,L-Lipoic acid, methanol (HPLC grade), trifluoroacetic acid (TFA, amino acid sequencing grade), chloroform (HPLC grade) and 1,4-dioxane (HPLC grade) were obtained from Wako (Osaka, Japan). PAB was from Kanto Chemical (Tokyo, Japan). Thionyl chloride and oxalyl chloride were purchased from Nakarai (Kyoto, Japan).

The substrate (LPAB) was pepared from lipoic acid and PAB. The purity of the synthesized product was assessed by the HPLC method described below. Synthesized LPAB was dissolved in chloroform and stored. Before use, an aliquot of

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TABLE I

TYPICAL ELUTION PROGRAMME ROUTINELY USED IN THIS STUDY

Proportioning valves A and B were used. The column back-pressure ranged from 84 to 168 bar.

Time (min)	Flow-rate (ml/min)	Solvent A (%)	Solvent B (%)
Initial	1.00	100	0
1.00	1.00	70	30
11.00	1.00	0	100
12.00	1.00	0	100
12.01	1.00	100	0

the stock solution was pipetted and dried under nitrogen. Then, an appropriate volume of 0.1 *M* sodium phosphate buffer (pH 7.0) containing 10 m*M* 2-mercaptoethanol was added, and the resulting solution was used as a substrate.

High-performance liquid chromatography

The liquid chromatograph was a Waters 600 equipped with a sample injector (U6K) and a fluorimeter (F-3000, Hitachi, Japan). The gradient programme is indicated in Table I. Determination of PAB and LPAB was performed by measuring the corresponding peak heights manually.

The column was a Nucleosil 3C18 (50×4.0 mm I.D.), manually packed as described before [3], from Macherey–Nagel (Düren, F.R.G.) used with a guard column (Develosil ODS, Nomura, Aichi, Japan; 50×4.0 mm I.D.).

Simultaneous determination of PAB and LPAB was carried out using a linear gradient from 100% solvent A (aqueous 0.1% TFA) to 100% solvent B (methanol). Total analysis time was 20 min. PAB and LPAB were detected by measuring their intrinsic fluorescence with an excitation wavelength of 276 nm and emission wavelength of 340 nm.

Enzyme assay

Partially purified enzyme was prepared from 50 ml of dialysed serum against 0.1 *M* sodium phosphate buffer (pH 6.0). Dialysed serum was separated on a DEAE-Sephacel column (40×1.5 cm I.D.) by a linear gradient from 0.1 to 0.3 *M* sodium phosphate buffer (pH 6.0). About fifteen-fold purification was achieved by DEAE chromatography and used as partially purified enzyme.

The enzyme solution (0.02 ml, serum and partially purified enzyme) was added to 0.08 ml of the substrate solution as described above. The conditions for the assay were essentially the same as previously described [1]. After incubation at 37°C for appropriate time, the reaction was stopped by boiling for 1 min. Then, 0.2 ml of methanol was added to the boiled reaction mixture. After centrifugation for 5 min at 1500 *g*, a clear supernatant solution was obtained, and a portion (10 μl) was injected into the HPLC system. Protein was determined by the method of Lowry et al. [4].

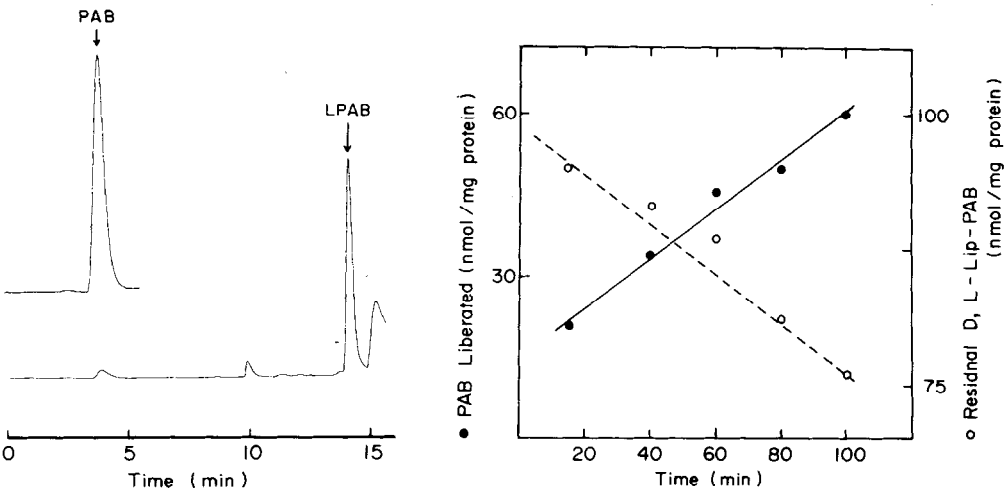

Fig. 1. Chromatogram of a serum sample. For analytical conditions see Experimental. The PAB peak is on an enlarged scale (thirty-fold).

Fig. 2. Hydrolysis of LPAB in a partially purified sample as a function of time. Conditions are as described in Experimental; protein concentration, 1.68 mg/ml; substrate concentration, 47 μM.

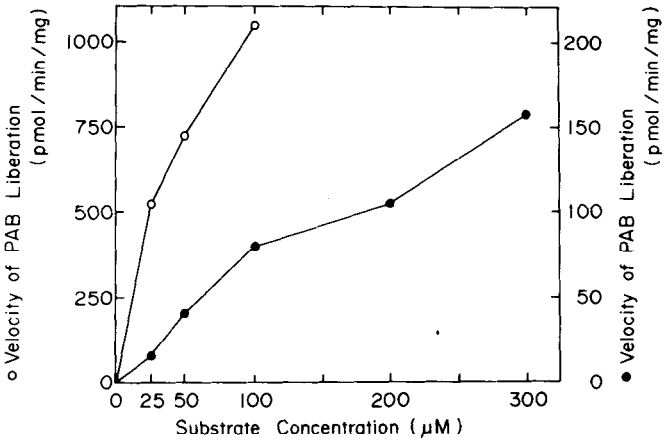

Fig. 3. Effect of the concentration of LPAB (substrate) on the rate of hydrolysis; assay conditions as described in Experimental. LPAB hydrolase activity was measured after 2 h incubation. Open circles, partially purified enzyme of 1.68 mg/ml; closed circles, serum sample of 55 mg/ml.

RESULTS AND DISCUSSION

When a Nucleosil 3C18 column and the gradient programme shown in Table I were used, the two standards (PAB and LPAB) were eluted at 3.8 and 14.0 min, respectively. The relationship between the amounts of PAB and LPAB injected and the peak heights was linear in the concentration range 10–1500 pmol.

We applied this method to serum and a partially purified enzyme solution. A typical example of the chromatographic separation is shown in Fig. 1. The coef-

ficients of variation (C.V.) for determination of PAB and LPAB were similar to these previously described (C.V. less than 2%) [1].

The time-course of hydrolysis of the substrate (LPAB) is as shown in Fig. 2. PAB and LPAB increased linearly and decreased linearly in a stoichiometric manner. We always observed that the increase of PAB was relatively higher than the decrease of LPAB. This might be a result of poor solubility of LPAB in the aqueous reaction mixture.

The effect of the substrate concentration on the rate of the enzyme reaction was studied, and the results are shown in Fig. 3. We found that the rate of the enzyme reaction in serum increased considerably with increasing substrate concentration. The substrate is apparently not soluble in the buffer at concentrations above 100 μM; thus a partially purified enzyme was tested at the lower substrate concentration. Our results indicate that the enzyme activity can be determined at a fixed substrate concentration.

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