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High-performance liquid chromatographic determination of four kinds of biotin-containing carboxylase

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ABSTRACT

A convenient and reliable reversed-phase liquid chromatographic assay for four kinds of carboxylase, i.e. pyruvate carboxylase, acetyl-coenzyme A carboxylase, propionyl-coenzyme A carboxylase, and 3-methylcrotonyl-coenzyme A carboxylase, was developed. These are all biotin enzymes, which contain covalently bound biotin as a co-factor (co-enzyme). This chromatographic method, which is free from interferences, gives a stoichiometric and precise assay for these enzymes. It is non-radioisotopic, which makes it suitable for clinical laboratories. The assay time required for one sample is ca. 30 min. The method was satisfactorily applied to guinea-pig kidney homogenates after a simple pretreatment with acid.

INTRODUCTION

Carboxylases ligate carbon dioxide by consuming adenosine triphosphate (ATP) to each of four kinds of substrate, i.e. pyruvic acid, acetyl-coenzyme A (acetyl-CoA), propionyl-CoA, and 3-methylcrotonyl-CoA (for reviews, see refs. 1–3). These four enzymes are present in eukaryotes and are presumed to contain biotin as a covalently bound co-enzyme and magnesium ions as a co-factor metal. The enzymes are considered to play key roles in lipogenesis (fatty acid biosynthesis) by acetyl-CoA carboxylase, gluconeogenesis by pyruvate carbox-

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ylase, amino acid (isoleucine, threonine, and methionine) and odd-numbered fatty acid metabolism by propionyl-CoA carboxylase and amino acid (leucine) metabolism by 3-methylcrotonyl-CoA carboxylase.

These enzymes have been assayed by determining the incorporation of radioisotopic carbon dioxide into the respective substrates or by the colorimetric ADP assay method [1,2]. The latter cannot be applied to tissue homogenates that contain ATPases. The isotope method is applicable to homogenates, but the handling of dangerous radioisotopes usually requires a special room, and pretreatment of isotopically labelled compounds and radiation counting are relatively time-consuming. Furthermore, carbon dioxide is liberated to the surrounding air and may cause air pollution. Assaying only the product after incorporation of the isotopic substrate may not be stoichiometric.

We have previously developed stoichiometric high-performance liquid chromatographic (HPLC) assays for biotinidase [4] and lipoamidase [5], which analyse simultaneously the increase of the product and the decrease of the substrate. We have now developed a reliable and convenient reversed-phase HPLC method for four kinds of biotin-containing carboxylase. The method is stoichiometric, requires a single octadecylsilane column and requires an analysis time of only 20–30 min per assay. The method was satisfactorily applied to the tissue of guinea-pig. Furthermore, because of the relatively short analysis time, this method is expected to be applicable to the enzyme purification.

EXPERIMENTAL

Chemicals and reagents

Pyruvic acid and oxaloacetic acid (OAA) were obtained from Wako (Osaka, Japan). Propionyl-CoA, methylmalonyl-CoA, 3-methylcrotonyl-CoA, acetyl-CoA, malonyl-CoA, malic dehydrogenase (MDH; L-malate: NAD^+ oxidoreductase; EC 1.1.1.37) from bovine heart, pyruvate carboxylase (PC; EC 6.4.1.1) from bovine liver, hen egg-white avidin and L-malic acid disodium salt were purchased from Sigma (St. Louis, MO, U.S.A.). Reduced-form nicotinamide-adenine dinucleotide (NADH) was from Oriental Kobo (Tokyo, Japan). ATP was from Boehringer-Mannheim Yamanouch (Tokyo, Japan). 2-Mercaptoethanol (ME), Nomidet P-40 (NP-40), ethylenediaminetetraacetic acid (EDTA), methanol (HPLC grade), sodium sulphate, and sodium bicarbonate were from Nakalai Tesque (Kyoto, Japan).

Specimens

Guinea-pig kidney homogenates were employed as the enzyme source. Kidney homogenates were prepared in 0.32 *M* sucrose solution containing 1 m*M* sodium phosphate buffer (pH 7.0). Tissues were weighed and cut into pieces with a scalpel, and a ten-fold volume (with respect to wet weight) of the sucrose solution was added. They were homogenized at 4°C with a Potter–El-

vehjem homogenizer, and the homogenates were stored at −20°C until use. Usually the protein concentration in the homogenates was ca. 10 mg of protein per ml.

Enzyme assay conditions

Acetyl-CoA carboxylase (ACC; EC 6.4.1.2), propionyl-CoA carboxylase (PCC; E.C. 6.4.1.3) and 3-methylcrotonyl-CoA carboxylase (MCC; EC 6.4.1.4) were assayed essentially as described by Hunaiti and Kolattukudy [6]. The reaction buffer was composed of 80 m*M* potassium phosphate (pH 7.0), 3 m*M* ATP, 5 m*M* magnesium chloride, 30 m*M* sodium bicarbonate, 0.5 m*M* substrates (acetyl-CoA, propionyl-CoA or methylcrotonyl-CoA), and enzyme solution in a total volume of 0.04 ml. The enzyme concentrations used were 0.05 mg of protein–reaction mixture for PCC and ACC, and 0.01 mg of protein–reaction mixture for MCC. The reaction was started by adding the enzyme solution, allowed to proceed at 37°C for 20 min and stopped by addition of 0.08 ml of 6 *M* hydrochloric acid. After centrifugation at 1500 *g* for 5 min, the supernatant was recovered. A portion (0.02 ml) of the supernatant solution was injected into the HPLC system.

Pyruvate carboxylase (PC; EC 6.4.1.1) was assayed essentially according to the method Chandler and Ballard [7]. The reaction mixture contained 50 m*M* Tris–HCl (pH 7.2), 5 m*M* ATP, 5 m*M* magnesium sulphate, 1 m*M* pyruvate, 0.5 m*M* EDTA, 10 m*M* potassium bicarbonate, 0.7 m*M* NADH, 6 μg of MDH, 0.1 m*M* acetyl-CoA (as an activator) and the enzyme solution (0.01 mg of protein). The reaction volume was 0.05 ml. The reaction was started by addition of the enzyme at 37°C. After 5 min, the reaction was stopped by adding 0.1 ml of 6 *M* hydrochloric acid. The solution was centrifuged at 1500 *g* for 5 min, and 0.01 ml of the supernatant was injected into the HPLC system.

High-performance liquid chromatography

The HPLC system consisted of an LKB 2150 HPLC pump with a 2152 LC controller (Pharmacia-LKB, Uppsala, Sweden), a Model SSC-EIE-005 rotary switching valve injector with a 0.2-ml sample-holding loop (Senshu Scientific, Tokyo, Japan), a column oven (Hitachi 655A-52), and a Model 655A-21 variable-wavelength UV monitor (Hitachi, Tokyo, Japan). A single commercial reversed-phase octadecylsilane column of Nucleosil $5C_{18}$ (250 mm×4.6 mm I.D., Wako) was used for the four kinds of carboxylase. Compounds containing CoA were detected at 260 nm, and pyruvate and OAA were detected at 210 nm. The purity of pyruvate, malate and OAA was assessed with a refractive index detector (Model ERC-7521, Erma, Tokyo, Japan).

MCC assay conditions

The substrate (3-methylcrotonyl-CoA) and the putative product (3-methylglutaconyl-CoA) were separated by a linear gradient from solvent A (0.1 *M* sodium phosphate buffer, pH 2.1) to solvent B (methanol–solvent A, 80:20,

v/v) in 15 min. The flow-rate was 1.5 ml/min. The column inlet pressure was 90–130 kg/cm^2, and the column temperature was 45 °C. The total assay time was 20 min per sample.

PC assay conditions

The substrate (pyruvate) and the product (OAA) were assayed by isocratic elution using a mobile phase of high ionic strength (salting-out chromatography). However, because OAA quickly diminished in the reaction mixture containing MDH, the reduction of pyruvate was monitored. The mobile phase was 0.1 *M* sodium phosphate (pH 2.1) containing 1.0 *M* sodium sulphate. The flow-rate was 1.0 ml/min. After the analysis, the pump was washed with water. The total assay time was 30 min per sample.

ACC assay conditions

The substrate (acetyl-CoA) and the product (malonyl-CoA) were assayed under the same conditions as MCC. The total assay time for one sample was 20 min.

PCC assay conditions

The substrate (propionyl-CoA) and product (methylmalonyl-CoA) were assayed by a linear gradient by the same method as the MCC assay. The total assay time for one sample was 20 min.

Omission tests

The requirements for the carboxylation reaction are carbonate (one of the substrates), ATP and Mg^{II}; it will not proceed if any of these substances is absent. We thus examined whether the stoichiometric increase in product and decrease in substrate were related to the presence of these three compounds. A reaction mixture without ATP, Mg^{II} or carbonate was made, and the reaction rate was compared with that observed with that of a reaction mixture that contained the three components (complete reaction mixture).

Inhibition test by avidin

Because all four carboxylases contain biotin as a co-factor in the carboxylatin reaction, specific inhibition of the carboxylation reaction should occur upon addition of avidin. The specific inhibition by avidin was tested by preincubating avidin with the enzyme solution at a concentration of 1 mg of avidin per ml of preincubation mixture. After the incubation at 37 °C for 10 min, 0.004 ml of the avidin-treated enzyme was added to the reaction mixture. Other reaction conditions were the same as described above.

Protein assay

The protein concentration was determined by using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Since the omission of dangerous radioisotopes from the carboxylase assay is desirable, if routine assays are to be performed in the laboratory without special precautions, we developed HPLC assay methods for four kinds of biotin-containing carboxylase by using a single ODS column. As shown in Figs. 1–4, the substrates and the products for all four carboxylases can be separated with an acidic phosphate buffer system. CoA-containing substrates and products were separated by gradient elution, and the assay time was less than 20 min. The use of acidic aqueous 0.1% trifluoroacetic acid as a mobile phase caused peak tailing for CoA-containing compounds. The product of MCC commercially was not available but it was estimated as the stoichiometrically increasing peak in accordance with substrate disappearance (Fig. 1). Thus the MCC assay was performed by measuring the substrate disappearance.

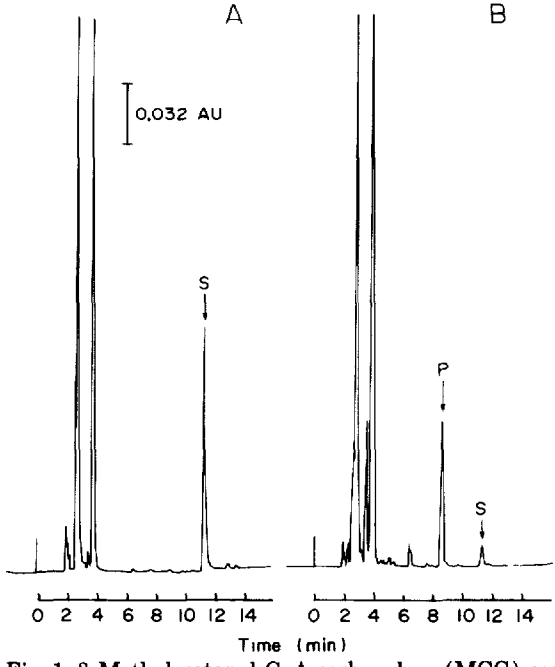

Fig. 1. 3-Methylcrotonyl-CoA carboxylase (MCC) assay. Guinea-pig kidney homogenates were used. (A) Boiled homogenates (control). (B) Homogenates (sample). Peaks: S = substrate, 3-methylcrotonyl-CoA; P = product, putative 3-methylglutaconyl-CoA. Conditions were as described in Experimental.

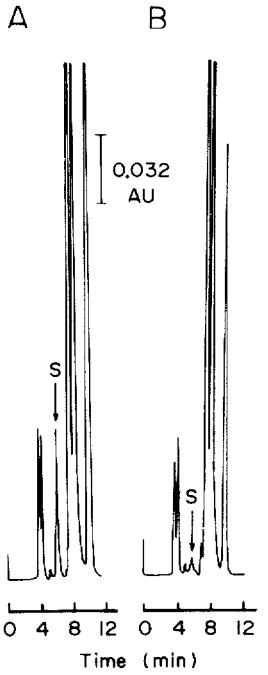

Fig. 2. Pyruvate carboxylase (PC) assay. Guinea-pig kidney homogenates were used. (A) Boiled homogenates (control). (B) Homogenates (sample). Peak: S = substrate, pyruvate. Conditions were as described in Experimental.

An isocratic pyruvate analysis method, which uses a mobile phase containing a high (1 *M*) concentration of sodium sulphate, was employed for the PC assay. In this system, the product of OAA was eluted close to the injection point. However, the assay reaction mixture containing MDH caused the conversion of OAA into malic acid. The decrease in substrate (pyruvate) was only detectable in the usually employed method [7] containing MDH. Further inclusion of MDH and NADH was found to be essential for the PC reaction. Malic and pyruvic acids eluted at retention times of 5.2 and 6.0 min. A stoichiometric increase in malic acid was observed. However, the absorption of pyruvate at 210 nm was ca. twenty-fold higher than that of malate, therefore the pyruvate was usually measured. The PC assay of homogenates required 30 min, since the interfering peaks were eluted at ca. 18 and 25 min.

Assays for ACC and PCC were possible under the assay conditions for MCC (Figs. 3 and 4). The use of an acidic phosphate buffer and methanol was very economical and gave symmetrical peaks and reproducible results: the coefficients of variation (C.V.) of the retention times of the seven compounds were less than 2.0%. Stoichiometric increase of the product and decrease of the substrate were observed for the MCC, ACC and PCC assay as already described

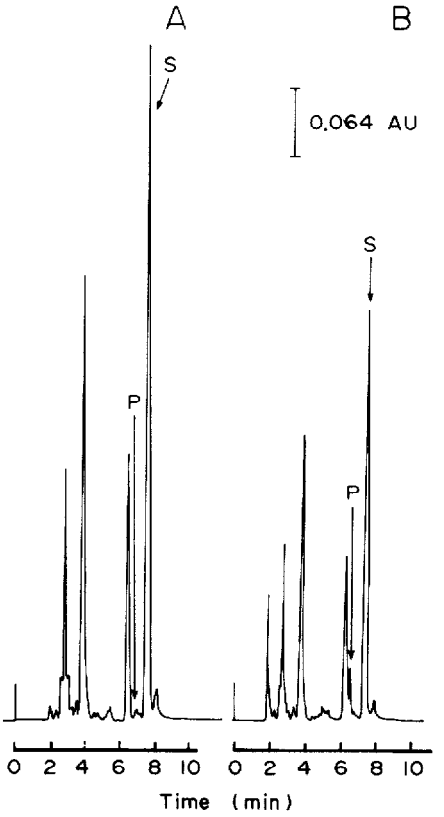

Fig. 3. Acetyl-CoA carboxylase (ACC) assay. Kidney homogenates were used. (A) Boiled homogenates (control). (B) Homogenates (sample). Peaks: S = substrate, acetyl-CoA; P = product, malonyl-CoA. Conditions were as described in Experimental.

[4,5]. A linear reaction time was obtained over 30 min for CoA carboxylases and over 10 min for PC. C.V. for the measurement of the products and substrates for CoA compounds were within 2% ($n=5$), and the C.V. for pyruvate was within 5% ($n=5$).

Carboxylases require a minimum of four compounds in the reaction mixture, i.e. substrate, carbon dioxide, ATP, and Mg^{II}. The omission test was performed for the MCC assay, and the results are shown in Table I. Omission of carbonate resulted in 18% residual activity. This may be due to the difficulty of omitting the endogeneous carbon dioxide. Addition of GTP instead of ATP into the reaction mixture could not induce the reaction to proceed. The other three carboxylases gave essentially similar results (data not shown), which indicates that the stoichiometric changes in peak heights of the substrate and product were caused by the carboxylate-induced reaction.

Avidin is a specific binding protein for biotin, and specific inhibition for biotin-containing carboxylase was induced by this protein [1–3]. Specific in-

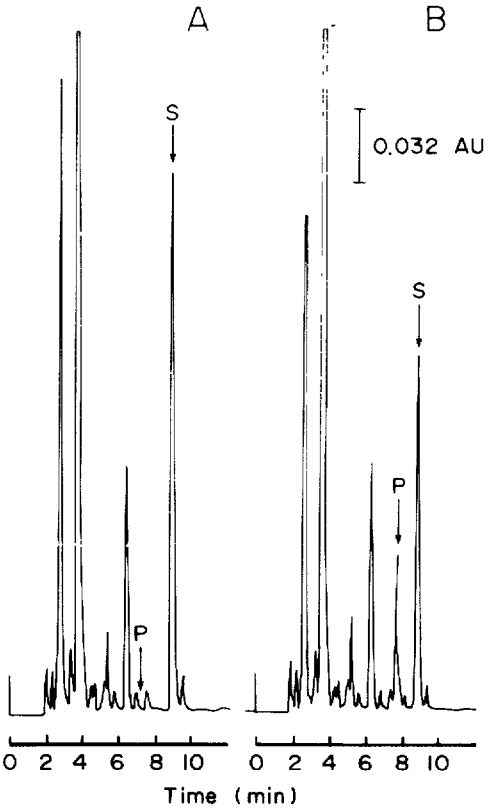

Fig. 4. Propionyl-CoA carboxylase (PCC) assay. (A) Boiled homogenates (control). (B) Homogenates (sample). Peaks: S = substrate, propionyl-CoA; P = product, methylmalonyl-CoA. Conditions were as described in Experimental.

TABLE I

REQUIREMENTS FOR MCC ACTIVITY IN GUINEA-PIG KIDNEY HOMOGENATES

Reaction mixture	Relative activity (%)
Complete	100[a]
Carbonate absent	13
Substrate (methylcrotonyl-CoA) absent	0
ATP absent	0
Mg^{II} absent	0

[a]Specific activity was 0.92 nmol/min per mg of protein.

TABLE II

EFFECT OF AVIDIN ON FOUR KINDS OF BIOTIN-CONTAINING CARBOXYLASE

Guinea-pig kidney homogenates were used. Specific activities were: MCC, 0.92 nmol/min per mg or protein; PC, 65 nmol/min per mg of protein; ACC, 1.80 nmol/min per mg of protein; PCC, 1.56 nmol/min per mg of protein. Assay conditions were as described in Experimental.

Carboxylases	Residual activity (%)	
	Without avidin	With avidin[a]
MCC	100	19
PC[b]	100	23
ACC	100	0
PCC	100	0

[a]Final concentration of avidin 0.1 mg/ml.
[b]Guinea-pig kidney S3 (supernatant fraction of homogenates obtained by ultracentrifugation at 100 000 *g* for 60 min) was used.

hibition by avidin on these carboxylases in guinea-pig kidney homogenates was tested, and the results are shown in Table II. Specific activities observed in guinea-pig kidney homogenates are also shown in Table II. ACC and PCC were found to be completely inhibited by the presence of avidin. Inhibition of MCC and PC by avidin was not complete (ca. 80% inhibition), This might be due to the inaccessibility of avidin to the active biotin residue(s) in the case of these latter two enzymes. Demonstration of the specific avidin-inhibition also supported the view that a specific carboxylase assay was achieved by this HPLC method, and that the method is applicable to kidney homogenates.

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