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Determination of Acetoacetate in Plasma by a Combination of Enzymatic Decarboxylation and Head-Space Gas Chromatography

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Acetoacetate concentration in plasma was determined by head-space gas chromatography after decarboxylation by the use of acetoacetate decarboxylase, which was recently isolated from *Bacillus polymyxa* A-57 strain. Partial purification of acetoacetate decarboxylase solution was carried out by DEAE-cellulose column chromatography of the cell-free extract, which was prepared by ultrasonication. The properties of the enzyme were found to be particularly suitable for acetoacetate assay (optimum pH, 5.8; V_{max}, 230 μmol/min/mg; K_m, 5.9×10^{-4} M). Plasma was deproteinized by Somogyi's method. Acetone in the mildly basic supernatant was assayed by head-space gas chromatography. Acetoacetate was calculated by subtracting blank acetone from total acetone after enzymatic decarboxylation of acetoacetate. The minimal detectable concentration was 1 μM. This method gave better reproducibility ($CV = 2.0$—8.0%) and recovery (96.0—101.8%) than chemical decarboxylation with perchloric acid. Normal subjects ($n = 31$) showed plasma acetone levels of 7.2 ± 3.4 μM and acetoacetate levels of 22.5 ± 9.7 μM. Diabetic patients ($n = 44$), treated by diet control alone without drug therapy, gave plasma acetone levels of 8.1 ± 3.5 μM and acetoacetate levels of 25.0 ± 8.0 μM. There was no significant difference between the two groups.

Keywords——acetone; acetoacetate; ketone body; head-space gas chromatography; acetoacetate decarboxylase; *Bacillus polymyxa*; acetoacetate decarboxylation; diabetes mellitus

The determination of acetoacetate, one of the ketone bodies in blood, is important for the diagnosis of diabetes mellitus. Several methods have been developed for acetoacetate assay.[1] The strip-test method, using sodium nitroprusside,[1h,i] has been used conventionally in clinical laboratories; it is very simple and rapid, but is semiquantitative and cannot be applied to concentrations of less than 300 μM acetoacetate.

In spite of the high sensitivity of the enzymatic method using 3-hydroxybutyrate dehydrogenase, its reproducibility was not satisfactory at low concentrations (less than 100 μM) in our laboratory. Eriksson has reported a head-space gas chromatographic method which permits determination of micromolar concentrations of acetone, acetoacetate and 3-hydroxybutyrate.[1g] Since undesirable by-products arose due to the use of strong acid and heating, the reproducibility of the assay was not satisfactory. We describe here an improved method for determining acetone and acetoacetate using acetoacetate decarboxylase, an enzyme which was recently isolated from *Bacillus polymyxa* A-57 strain.

Experimental

Materials——Lithium acetoacetate was purchased from Sigma Chemical Co., U.S.A. Acetone and other chemicals were analytical reagent-grade products of Wako Pure Chemical Industries Ltd., Japan.

Gas Chromatography——Apparatus: Hitachi gas chromatograph, type 073 (Hitachi Ltd., Japan); glass column, 2 m × 3 mm i.d.; column packing, 10% PEG 600 Chromosorb WAW, 80/100 mesh; temperature, column oven 117 °C, injector and detector 140 °C; carrier gas, nitrogen; flow rate, 30 ml/min; hydrogen flame ionization detector (FID).

Preparation of Crude Acetoacetate Decarboxylase Solution——1) Source of Enzyme: *Bacillus polymyxa* A-57 strain was selected from 90 strains of acetone-producing *Bacillus* sp. by screening for the enzyme activity using Ketostix (Ames Division, Miles-Sankyo Co., Ltd., Japan). The A-57 strain was cultured in maltose medium (malt extract [Difco] 10%, ammonium sulfate 0.1%; pH 7.2) at 28 °C for 36 h. The bacterial mass was harvested by centrifugation and was then lyophilized. The yield was 0.8 g/l.

2) Cell-Free Extract: 20 ml of 0.1 M phosphate buffer (pH 5.8) and 0.5 ml of Triton X-100 were added to 500 mg of this lyophilized cell powder. The mixture was sonicated at 20 kHz for 30 min by means of a sonifier (Tomy model UR-200p, Tomy Seiko Ltd., Japan) equipped with a micro tip, and was then centrifuged for 30 min at 15000 × *g*.

3) Partial Purification of the Enzyme: Cell-free extract (8 ml) was chromatographed on a DEAE-cellulose column (150 × 15 mm i.d.). Fractions having acetoacetate decarboxylase activity were combined and the mixture was concentrated to 4 ml by means of a collodion bag (Sartorius membrane filter, SM-13200, Sartorius GmbH, West Germany) and the concentrated enzyme solution was used. Purification procedures were performed at room temperature.

4) Assay of Acetoacetate Decarboxylase Activity: The activity was assayed on the basis of either the decrease of absorbance of acetoacetate at 210 nm or the increase of acetone found by head-space gas chromatography. One unit (U) of activity was defined as the amount of enzyme required to produce 1 μmol of acetone per min from acetoacetate. Protein was assayed by Lowry's method.[2]

Determination of Acetone and Acetoacetate in Plasma——1) Preparation of Sample: Whole venous blood was obtained from normal subjects and from diabetic patients, treated by diet control alone without drug therapy. Blood samples (2 ml) were collected between 1.5 and 3 h after a meal, pipetted into heparinized tubes, and centrifuged immediately to separate the plasma. For deproteinization, by Somogyi's method,[3] 1 ml of 1% barium hydroxide solution was added to 0.5 ml of plasma with gentle mixing. Then 1 ml of 1% zinc sulfate solution containing 0.0005% methyl ethyl ketone as an internal standard (IS) was added and mixed. The precipitate was centrifuged for 15 min at 2800 × *g*.

2) Assay of Acetone: 0.5 ml of the supernatant of the deproteinized plasma was incubated in a 10 ml sealed vial for 15 min at 50 °C, and then 1 ml of gas from the vial was injected into a gas chromatograph.

3) Assay of Acetoacetate: 0.1 ml of 0.2 M phosphate buffer (pH 5.8) and 0.1 ml of the enzyme solution were added to 0.5 ml of the deproteinized sample in a vial, and the vial was sealed. After incubation for 30 min at 50 °C, 1 ml of the gas in the vial was injected into a gas chromatograph. Acetoacetate value was calculated by subtracting blank acetone from total acetone.

4) Decarboxylation of Acetoacetate by Perchloric Acid: 0.2 ml of 3 N perchloric acid was added to 0.5 ml of supernatant in a vial, and the mixture was incubated for 1 h at 75 °C to induce decarboxylation. After cooling, the total acetone was determined as described above.

Except for the incubation, all procedures of sample preparation and assay were carried out in an ice bath. Glucose was assayed by the glucose oxidase method (Beckman glucose analyzer).

Results and Discussion

Acetoacetate decarboxylase (EC 4.1.1.4) was first isolated from *Clostridium acetobutyricum* by Davies[4] and was subsequently crystallized by Westheimer *et al.*[5] We attempted to separate acetoacetate decarboxylase from both *C. acetobutyricum* IFO 3346 and IFO 13948, but their enzyme production levels were unsatisfactory. We then found *Bacillus polymyxa* A-57 strain, which produced a high yield of acetoacetate decarboxylase, by screening of microorganisms. This organism, which was isolated from a soil sample taken in Nishinomiya, Japan, is an aerobic, gram-variable, spore-forming, rod-shaped bacterium, and is considered to belong to the genus *Bacillus*. The morphological, cultural and physiological characteristics of the strain A-57 provided the basis for identification of the organism as *Bacillus polymyxa* according to Bergey's Manual of Determinative Bacteriology (the details of this identification will be reported elsewhere). The cell-free extract of A-57 strain was prepared by ultrasoni-

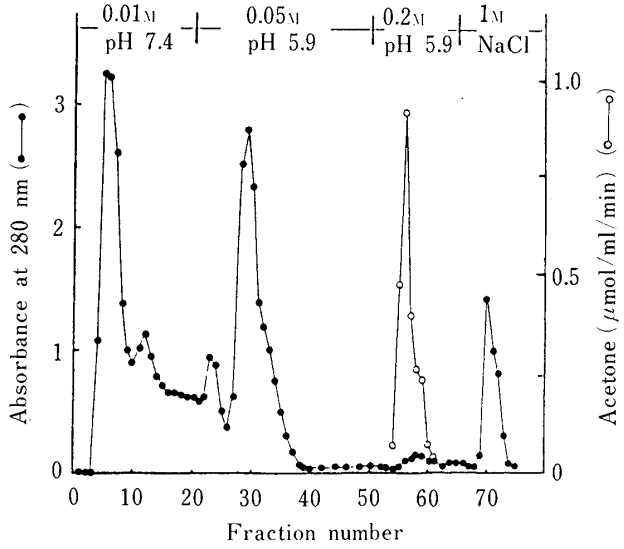

Fig. 1. Chromatography of Cell-Free Extract on a DEAE-Cellulose Column

Column, DEAE-cellulose (150 × 15 mm i.d.); sample, cell-free extract 8 ml; eluent (step-wise), 0.01 M phosphate buffer (pH 7.4), 0.05 M phosphate buffer (pH 5.9), 0.2 M phosphate buffer (pH 5.9), and 1 M sodium chloride; flow rate, 0.8 ml/min; 4 ml/fraction. The decarboxylase activity of each fraction was pre-checked by the use of Ketostix, and then the exact activity of the active fractions was determined by head-space gas chromatography.

TABLE I. Purification of Acetoacetate Decarboxylase in Cell-Free Extract from *Bacillus polymyxa*

Sample	Total protein (mg)	Specific activity (μmol/min/mg)	Total activity (μmol/min)	Yield of total activity (%)
Cell-free extract	28.4	8.5	241.4	100
DEAE-cellulose chromatographed (0.2 M fr.)	0.33	230.0	75.9	31

cation in the presence of detergent (Triton X-100), and was partially purified by DEAE-cellulose column chromatography. Step-wise elution, as shown in Fig. 1, varying the pH from 7.4 (step 1) to 5.9 (step 2), gave excellent resolution of the active enzyme fraction. Partial purification of the cell-free extract increased the specific activity 27-fold over the original activity (8.5 μmol/min/mg), while 31% (75.9 U) of the original activity remained (Table I). No acetone was present in the purified sample, although it was present in the cell-free extract.

Properties of Acetoacetate Decarboxylase from *Bacillus polymyxa*

The enzyme showed an optimum pH of 5.8, V_{max} of 230 μmol/min/mg, and K_m of 5.9×10^{-4} M. The enzyme was stable between pH 4 and 11 when incubated for 30 min at 37 °C. At pH 3, 75% of the activity remained, but all activity was lost below pH 2 and over pH 12. The enzyme activity was not decreased by incubation for 1 h at 60 °C, but it was reduced to 70% at 70 °C. The enzyme was completely inactivated by heating for 1 h at 80 °C. When the crude enzyme solution (pH 5.8) was refrigerated for 1 month at 4 °C, the activity was not decreased. Figure 2 shows the inhibitory effect of chloride and fluoride ions. Phosphate ions were non-inhibitory. These properties are the same as those of the enzyme from *C. acetobutyricum*.[6] It is presumed that the chloride ions in plasma (0.02 M in the solution) may have a minor inhibitory effect, but it is not enough to disturb the determination of acetoacetate in practice.

Application of the Enzyme for Determination of Acetoacetate

The time course of decarboxylation of acetoacetate is shown in Fig. 3. The enzymatic decarboxylation was completed in about 15 min using 0.7 U of enzyme. When plasma

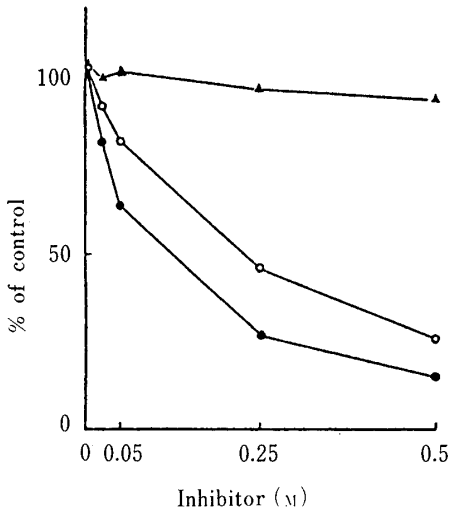

Fig. 2. Inhibitory Effect of Several Anions on Enzymatic Decarboxylation

The activities were assayed by measuring the decrease of absorbance at 210 nm at various concentrations of inhibitors and 0.05 M phosphate buffer (pH 5.8). Acetoacetate concentration: 1.9 mM. ▲, Sodium phosphate buffer (pH 5.8); ○, sodium fluoride; ●, sodium chloride.

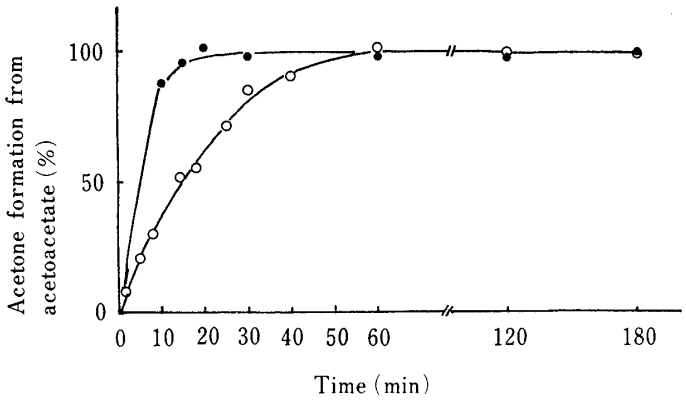

Fig. 3. Time Course of Acetone Formation from Acetoacetate by Enzymatic Decarboxylation

Decarboxylation was carried out at 50 °C for various incubation periods in medium containing 1 mM acetoacetate and either 0.7 U (●) or 0.12 U (○) of the enzyme.

acetoacetate was to be determined with 0.7 U of the crude enzyme solution, it was assumed that a 30 min incubation time was long enough for the decarboxylation of acetoacetate and the equilibrium formation of acetone in the vial. A linear relationship was obtained between the concentration of acetoacetate and the peak height ratio (acetoacetate/IS) over the range of 0—30 mM acetoacetate. Calibration curves for blank acetone and total acetone after acetoacetate decarboxylation were drawn separately. The minimal detectable concentration of acetoacetate was 1 μM. Spontaneous conversion of acetoacetate to acetone without the enzyme or acid was about 1% of acetoacetate concentration, and this conversion was considered to be negligible. The acetone peak decreased with increasing perchloric acid concentration (Fig. 4). The presence of perchloric acid reduced the volatility of acetone in aqueous solution, while phosphate buffer (pH 5.8) increased it.

Figure 5 shows chromatograms of volatile compounds in plasma from a diabetic patient. When acetoacetate decarboxylation was done by perchloric acid treatment, the chromatogram showed a peak interfering with the acetone peak. When enzymatic decarboxylation was used, there was no interference, and the acetone and IS peaks were about 1.5 times higher than those obtained in the perchloric acid method. Reproducibility and recovery of plasma acetoacetate in the enzymatic and perchloric acid methods are shown in Table II. Table III shows acetone, acetoacetate and glucose levels determined in plasma from normal subjects

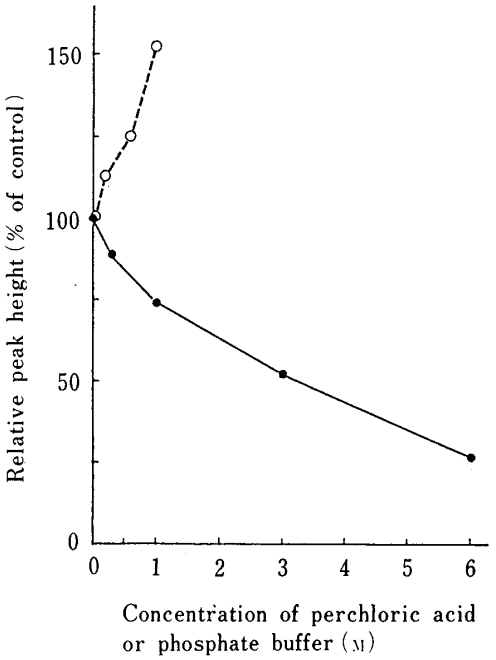

Fig. 4. Effect of Concentrations of Perchloric Acid and Phosphate Buffer on Acetone Peak Height in the Gas Chromatogram

Each point shows the mean value of three determinations. Acetone concentration, 1 mM; ○, phosphate buffer (pH 5.8); ●, perchloric acid.

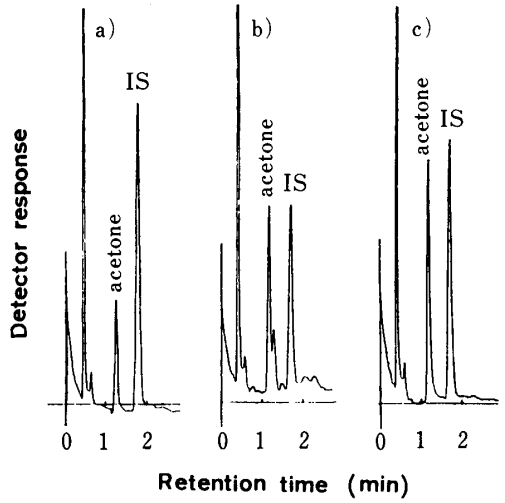

Fig. 5. Chromatograms of Volatile Compounds in Plasma from a Diabetic Patient

a) untreated; b) treated with perchloric acid; c) treated with acetoacetate decarboxylase.

TABLE II. Comparison of Plasma Acetoacetate Assay by Enzymatic and Perchloric Acid Methods

Method of decarboxylation	n	Acetoacetate (mean±S.D., μM)			Recovery (mean=S.D., %)	CV (%)
		Plasma	Added	Found		
Enzymatic	20	3.3	10.0	12.9±0.8	96.0±7.7	8.0
	20	4.4	50.0	55.3±2.1	101.8±4.1	4.0
	20	4.4	200.0	201.7±4.1	98.7±2.0	2.0
Chemical	20	10.0	50.0	56.7±3.6	93.5±7.1	7.6
(Perchloric acid)	16	10.0	200.0	224.6±8.6	107.3±5.3	4.9

TABLE III. Concentrations of Acetone, Acetoacetate and Glucose in Plasma of Normal Subjects and Diabetic Patients with Dietary Treatment

Subject	n	Acetone (μM)	Acetoacetate (μM)	Glucose (mg/100 ml)
Normal	31	7.2±3.4	22.5±9.7	94.5±8.3
Diabetic	44	8.1±3.5[a)]	25.0±8.0[b)]	130.0±60.7[c)]

a) $p>0.1$, *b)* $p>0.2$, *c)* $p>0.01$ (statistical comparison between normal and diabetic subjects was done by using Student's *t*-test).

and diabetic patients with dietary control treatment by the enzymatic decarboxylation method. Plasma acetone levels in normal subjects and non-ketotic diabetic patients have not been determined previously. The mean ratio of plasma acetone to acetoacetate was about 1 : 3

in both normals and diabetics (the range in both was from 1 : 8.3 to 1 : 1.2). So far, various normal values of acetoacetate have been reported by several investigators.[1b-d,7] The normal value (mean ± S.D.) of acetoacetate found by the present method was 22.5 ± 9.7 μM. This is comparable to the value (26 ± 11 μM) given by Hansen and Freier.[7c] No significant difference was observed between the acetone or acetoacetate levels of normal subjects and diabetic patients with dietary control treatment ($p > 0.1$).

In conclusion, acetoacetate decarboxylase was found to be suitable for use in an assay designed to measure acetoacetate in plasma by head-space gas chromatography. This enzyme was found in *Bacillus polymyxa* A-57 strain, which had been isolated from soil. The simple-to-prepare crude enzyme gave satisfactory results in the plasma acetoacetate assay. The enzymatic decarboxylation method does not have the disadvantages of the perchloric acid method: namely, the decrease of the acetone peak and the formation of undesirable by-products. Further, it offers satisfactory reproducibility and recovery of low concentrations of acetoacetate (less than 100 μM). No significant difference was found between normal subjects and diabetic patients on a controlled diet with respect to the levels of acetone and acetoacetate in plasma by using this method.

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