Practical Assay Method of Cytosolic Acetoacetyl-CoA Thiolase by Rapid Release of Cytosolic Enzymes from Cultured Lymphocytes Using Digitonin

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WATANABE, H., YAMAGUCHI, S., KIMURA, M., WAKAZONO, A., SONG, X-Q., FUKAO, T., ORII, T. and HASHIMOTO, T. Practical Assay Method of Cytosolic Acetoacetyl-CoA Thiolase by Rapid Release of Cytosolic Enzymes from Cultured Lymphocytes Using Digitonin. Tohoku J. Exp. Med., 1998, 184 (1), 29-38 —— We designed a simple approach to determine cytosolic acetoacetyl-CoA thiolase (CT) activity for differential diagnosis of ketone body catabolic defects, using rapid cell-subfractionation of cultured lymphocytes with digitonin. Efficiency of cell subfractionation was determined by measurement of lactate dehydrogenase and citrate synthetase as marker enzymes for cytosol and organelle fractions, respectively, and confirmed by immunotitration and immunoblotting using antibodies against cytosolic and mitochondrial thiolases, respectively. In the condition of best separation taken in the presence of 1 mg/ml digitonin, acetoacetyl-CoA thiolase activities in the presence of K+ ion in the cytosol and organelle fractions were 138.3±39.2 and 84.0±16.2 nmol/min/ml, respectively. The thiolase activity in the organelle fraction was doubled by the presence of K+ ion, whereas that in the cytosol fraction was not affected. The thiolase activity in the organelle fraction was reduced by the treatment of anti-mitochondrial acetoacetyl-CoA thiolase (T2) antibody but not by anti-CT antibody. On the other hand, that in the cytosol fraction was significantly decreased by anti-CT antibody but not by anti-T2 antibody. These data suggested that T2 was collected in the organelle fraction, and that CT activity could be assessed by measurement of the thiolase activity in the cytosolic fraction. Succinyl-CoA: 3-ketoacid CoA transferase (SCOT), whose defect is the third inherited disorder of ketone body catabolism, was collected in the organelle fraction. Hence, this method will prove to be useful for accurate assessment of defects of CT as well as T2 or SCOT, all involved in

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Ketoacidosis is not a rare condition in childhood, and often called "physiologic ketosis in childhood". Indeed, many diseases can potentially cause ketoacidosis: a) ketosis secondarily followed by miscellaneous acute illness, including infection, hyperpyrexia, or diarrhea, b) diabetes mellitus, c) inborn errors of organic acid metabolism, d) congenital defect in enzymes of glyconeogenesis, e) mitochondrial myopathies, or f) inherited ketone body catabolic defects.

As the inherited catabolic disorders of ketone body, three diseases are currently recognized: mitochondrial acetoacetyl-CoA thiolase (T2) deficiency, succinyl-CoA: 3-ketoacid CoA transferase (SCOT) deficiency, and cytosolic acetoacetyl-CoA thiolase (CT) deficiency. These might be missed in children who present severe and/or recurrent ketoacidosis.

T2-deficiency is a metabolic disorder of isoleucine and ketone body, which has been well known as beta-ketothiolase deficiency (Daum et al. 1971, 1973; Sweetman 1989), first described by Daum et al. (1971). The diagnosis of this disorder is based on the metabolic profiles of urinary organic acid analysis by GC/MS (Daum et al. 1973), or the evidence that acetoacetyl-CoA thiolase activity in tissue is not activated by the presence of K\(^+\) ion, because the K\(^+\) ion dependency is a specific property of T2.

SCOT-deficiency is caused by a defect in ketone body utilization. SCOT is the transfer of a CoA molecule from succinyl-CoA to acetoacetate. SCOT-deficiency was first described by Tildon and Cornblath (1972). In urinary organic acid analysis by GC/MS, however, no specific findings other than ketonuria are observed in such patients. The diagnosis, therefore, is made by enzyme assay, using fibroblasts or lymphocytes (Sakazaki et al. 1995).

CT plays a role in the formation of acetoacetyl-CoA from acetyl-CoA at the first step of cholesterol synthesis in the cytosol (Clinkenbeard et al. 1973). There have been only 2 patients with CT-deficiency reported (de Groot et al. 1977; Bennett et al. 1984). In these patients, the diagnosis was based on the reduction of acetoacetyl-CoA thiolase activity in fibroblasts. The enzymatic diagnosis of CT-deficiency in these reports, however, was not seemed exact enough and raises problems for the following reason.

Five thiolases have been identified in mammals: T2, mitochondrial medium-chain 3-ketoacyl-CoA thiolase (T1) and trifunctional protein (TFP), CT and peroxisomal 3-ketoacyl-CoA thiolase (PT) (Miyazawa et al. 1980). All but TFP have thiolase activity toward acetoacetyl-CoA as substrate. Therefore, total thiolase activity toward acetoacetyl-CoA in cell homogenate represent a whole activity derived from T2, T1, CT and PT. Moreover, the extent of acetoacetyl-
CoA thiolase activity in cell homogenates from normal controls can often vary (Yamaguchi et al. 1989; Fukao et al. 1996). The diagnosis of CT-deficiency deduced from the value of acetoacetyl-CoA thiolase activity is not tenable.

We report here a simple and practical assay method of CT activity for the detection of CT-deficiency and differential diagnosis of inherited ketone body catabolic defects by rapid release of cytosolic enzymes from cultured lymphoblasts or lymphocytes using digitonin.

**Materials and Methods**

**Materials.** Acetoacetyl-CoA, CoA, protein A sepharose, and digitonin were purchased from Sigma (Chemical, Co., St. Louis, MO, USA). The immunoblotting system was from Promega Biotec, Madison, WI. Rat T2 and T1, and human CT were purified according to the methods described elsewhere (Furuta et al. 1980; Miyazawa et al. 1980). Antibodies against T2, T1 and CT were raised in rabbits.

**Preparation of cultured cells.** EB virus-transformed B-lymphocytes (EB-lymphoblasts) and IL2-induced T-lymphocytes (IL2-lymphocytes) were prepared from about 5 to 10 ml of heparinized venous blood from healthy adult Japanese volunteers, as described (Steinaz et al. 1977; Koricheva et al. 1989). The EB-lymphoblasts and IL2-lymphocytes were cultured in 10% fetal caef serum/ RPMI 1640 medium.

**Cell subfractionation.** Cytosol enzymes in cells were released by the method of Mackall et al. (1979), with some modifications. In brief, cultured cells were washed twice with 150 mM NaCl and suspended in 0.25 M sucrose /0.1 M dithiothreitol /50 mM sodium phosphate (pH 8.0) (sucrose solution). These cells were then suspended in the sucrose solution and mixed with the same volume of 2 mg/ml of digitonin in the sucrose solution (final concentration of digitonin, 1 mg/ml) at 4°C for various time; 30 seconds, 1 minute, 3 minutes and 5 minutes, respectively. They were immediately centrifuged at 10,000 × g for 60 seconds at 4°C. The supernatant was taken as the cytosol fraction, and the pellet was resuspended in the sucrose solution as the organelle fraction including mitochondria, after washing twice with the sucrose solution.

**Enzyme assay.** Thiolase activities toward acetoacetyl-CoA and 3-ketoocanoyl-CoA as substrates were measured as described previously (Yamaguchi et al. 1989). Acetoacetyl-CoA thiolase activity was assayed in the presence and absence of 50 mM KCl. SCOT activity was measured according to the method by Sakazaki et al. (1995). Lactate dehydrogenase (LDH) and citrate synthetase (CS) as marker enzymes for the cytosol and mitochondria, respectively, were measured by the methods described in the literature (Lowry 1957; Srere 1969). The enzyme activities were expressed as “nmols/min/ml of initial suspension” to determine the enzyme distribution.

**Immunoblot analysis and immunotitration.** Immunoblotting was performed using antibodies against human CT, rat T2 and rat T1, according to the manufac-
ture's instruction of the immuno blotting system. In immunotitration experiments, 100 μl of the sample solution, whose protein concentration was adjusted to around 2 to 10 mg/ml, was incubated with 1 μl of the antibody solution, containing about 10-30 μg of IgG, at 25°C for 30 minutes. One μl of the antibody solution for each enzyme had been proved sufficient to immunotitrate each corresponding enzyme in the aliquots (data not shown). Fifty μl of 20% protein A sepharose suspended in saline (protein A solution) was added and the preparation was shaken gently at 25°C for 30 minutes. After centrifugation, the supernatants were used for enzyme assay.

Results

Release of cytosolic enzymes with digitonin

The efficiency of digitonin-subfractionation was determined in various cell density and various incubation time, monitoring CS and LDH activities. The optimum density was determined at 1.5 to 15×10⁷/ml. As shown in Fig. 1,

![Graph](image)

**Fig. 1.** Efficiency of cell subfractionation with digitonin using various densities of cells. LDH and CS were measured as cytosol and mitochondrial marker enzymes, respectively. Distribution of these enzymes were examined in the supernatant fraction (sup, ■) and pellet fraction (pt, □□) by %.
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subfractionation was likely to be more efficient at the density, 3.0 to 9.0 × 10⁷/ml under the condition of incubation time of 1 minute. The incubation time was compared between 30 seconds to 5 minutes at the cell density of 5 × 10⁷/ml. The thiolase activities at 1 minute, 2 minutes and 3 minutes were similar, whereas the thiolase activity in the cytosolic fraction at 30 seconds, and that in the organelle fraction at 5 minutes were significantly lower (data not shown). The results indicated that the incubation time between 1 minutes and 3 minutes gave the best separation in this condition. Hence, the following experiments were carried out at the cell density of around 5 × 10⁷/ml and the incubation time of 1 minute in the presence of 1 mg/ml of digitonin.

Distribution of thiolase activities

After cell subfractionation under the above condition, enzyme activities of acetoacetyl-CoA thiolase, 3-ketoacyl-CoA thiolase and SCOT as well as the respective marker enzymes, LDH and CS, in the cytosol (supernatant, sup) and organelle (pellet, pt) fractions, were determined. As shown in Table 1, LDH activities in the sup and pt fractions of the EB-lymphoblasts were 539.7 ± 49.4 and 74 ± 0.9 nmol/min/ml, respectively, whereas the CS activities were 102.5 ± 10.7 and 400.7 ± 9.4 nmol/min/ml, respectively. Acetoacetyl-CoA thiolase activities in the presence of K⁺ ion in the sup and the pt fractions were 138.3 ± 39.2 and 84.0 ± 16.2 nmol/min/ml, respectively. The ratio of the acetoacetyl-CoA thiolase activity in the presence and absence of K⁺ ion (+K/−K) in the cytosol was 1.0,

<table>
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<th></th>
<th>LDH</th>
<th>CS</th>
<th>Acetoacetyl-CoA thiolase</th>
<th>CS-CoA</th>
<th>SCOT</th>
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<td></td>
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<tr>
<td>Sup</td>
<td>539.7 ± 49.4</td>
<td>102.5 ± 10.7</td>
<td>138.3 ± 39.2</td>
<td>132.2 ± 35.7</td>
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<td>Pt</td>
<td>74.0 ± 0.9</td>
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<td>84.0 ± 16.2</td>
<td>36.7 ± 9.7</td>
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<td>Whole</td>
<td>583.6 ± 85.9</td>
<td>510.5 ± 44.8</td>
<td>253.0 ± 44.9</td>
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<td>Sup</td>
<td>497.4 ± 46.0</td>
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<td>119.7 ± 28.0</td>
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<td>Pt</td>
<td>64.7 ± 6.9</td>
<td>398.8 ± 13.0</td>
<td>76.2 ± 14.2</td>
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<tr>
<td>Whole</td>
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<td>455.7 ± 38.8</td>
<td>249.8 ± 35.2</td>
<td>124.9 ± 22.8</td>
<td>(2.0)</td>
</tr>
</tbody>
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(nmol/min/ml of initial suspension)

The activities are expressed nmol of each substrate catabolized/min/ml.

LDH, lactate dehydrogenase; CS, citrate synthase; +K and −K, acetoacetyl-CoA thiolase activity in the presence and absence of K⁺ ion, respectively; CS-CoA, 3-ketoacyl-CoA thiolase activity; SCOT, activity of succinyl-CoA; 3-ketoacid: CoA transferase; Sup, the supernatant fraction; Pt, the pellet fraction; Whole, whole cell homogenate before subfractionation.
whereas that in the organelle fraction was 2.3. These observations suggested that acetoacetyl-CoA thiolase activity derived from T2 that has K⁺ ion dependency, was predominant in the pt fraction. The K⁺ ion dependency of acetoacetyl-CoA thiolase activity in the sup fraction was nil. Namely, the thiolase collected in the organelle has K⁺ ion-dependency but not in the cytosol.

The thiolase activity toward 3-ketoocctanoyl-CoA, probably derived from T1, TFP and PT, and the SCOT activity were predominantly collected in the pt fraction but not in the sup fraction. Hence, CT was efficiently collected in the sup fraction, whereas T1, TFP or PT were predominant in the pt fraction. We also examined using IL2-lymphocytes, and obtained similar data or tendency, though the absolute values for each somewhat differed, as shown in Table 1.

**Immunotitration and immunoblot analysis**

The resultant solution of the sup and pt fractions after enzyme assay was used for immunotitration or immunoblot examination. Table 2 shows the results of the immunotitration experiments using EB-lymphoblasts. With the use of anti-CT antibody, acetoacetyl-CoA thiolase activity in the sup fraction decreased to less than 10% of that before antibody treatment, while it was not significantly affected by anti-T2 or T1 antibodies. The +K/−K ratio of 1.0 in the sup fraction was unchanged. The activity in the pt fraction was not significantly changed by the treatment of the anti-CT antibody. When the anti-T2 antibody was used, the activity toward acetoacetyl-CoA in the pt fraction decreased by about 40% and also the K⁺ ion-dependency disappeared. When both anti-T2 and T1 antibodies were used simultaneously, the activity decreased to about 10% before the treatment. These results indicated that CT was predominantly collected in the sup fraction, whereas T2 and T1 were in the pt fraction.

In immunoblot analysis, the cross reacted material (CRM) to CT was detected in lanes of homogenates of the human liver and whole cells, and the cytosol

| Table 2. Acetoacetyl-CoA thiolase activity after immunotitration with anti-CT, anti-T2 and anti-T1 antibodies |
|---------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sup                        | Pt              |                 |                 |                 |                 |
| +K | −K    | (+K/−K)       | +K   | −K    | (+K/−K)       |
| None | 122.8 | 118.6 (1.0) | 122.9 | 62.0 (2.0) |
| Anti CT | 10.8 | 10.2 (1.0) | 114.5 | 61.5 (1.9) |
| Anti T2 | 110.4 | 109.6 (1.0) | 26.4 | 24.5 (1.1) |
| Anti T1+T2 | 111.5 | 108.7 (1.0) | 3.6 | 3.2 (1.1) |

Thiolase activities were expressed as nmol of acetoacetyl-CoA breakdown/min/ml.

CT, cytosolic acetoacetyl-CoA thiolase; T2, mitochondrial acetoacetyl-CoA thiolase; T1, mitochondrial 3-ketoacyl-CoA thiolase. Other abbreviations were the same as described in Table 1 or the text.
Fig. 2. Immunoblots of cytosolic acetoacetyl-CoA thiolase (CT), mitochondrial acetoacetyl-CoA thiolase (T2) and mitochondrial 3-ketoacyl-CoA thiolase (T1).

Lane 1, pellet (organelle fraction, P) of human EB lymphocyte; lane 2, supernatant (cytosol fraction, S); lane 3, whole cell homogenates (W); and lane 4, human liver homogenates (Lv).

fraction (sup) of EB-lymphoblasts, as shown in Fig. 2. On the other hand, the bands for T2 and T1 were observed in lanes for the human liver, whole cell homogenates and the organelle fraction but not in lanes for the cytosol. These results were consistent with the data obtained by the enzyme assay and immunotitration experiments.

DISCUSSION

Inherited ketone body catabolic defects, including deficiencies of T2, SCOT and CT, are clinically characterized by intermittent ketoacidotic attacks and/or continuous ketonuria since infancy. Early detection and intervention of them are important to prevent neurological handicaps after such severe ketoacidosis.

Although T2-deficiency can be readily diagnosed by urinary organic acid analysis by GC/MS, detecting specific metabolites such as 2-methyl-3-hydroxybutyrate, tiglylglycine or 2-methylacetoacetate that are intermediates of isoleucine catabolism. In case of deficiencies of SCOT or CT, specific diagnosis by GC/MS alone may be impossible. While the enzymatic diagnosis of T2- and
SCOT-deficiencies using fibroblasts or lymphocytes is possible, that for CT-deficiency is not easy and has not been established up to now, because the value of acetoacetyl-CoA thiolase activity in cells represents the total activity of CT, T2, T1 or PT (Fukao et al. 1996) and often ranges widely (Yamaguchi et al. 1993).

Recently, Fukao et al. (1996), showed the distribution of each thiolase in cell homogenates by immunotitration experiment. This procedure, however, is not practical for the diagnosis of CT-deficiency, because it requires antibodies against thiolases and the procedure is somewhat complicated. In this study, we designed a practical assay method for evaluation of CT activity, and confirmed the efficiency of the method immunobiochemically. In practice, this method does not need such process with antibodies.

There are two patients reported previously as having CT deficiency. In their reports, they presented with developmental retardation, hypotonus, ataxia or choreoathetosis, and persistent increased excretion of ketone body since infancy (de Groot et al. 1977; Bennett et al. 1984). The diagnosis was based on the following results; one was the reduced value in the cytosol fraction of the biopsied liver after separation of mitochondria and cytosolic fractions on a DEAE sepharose column (de Groot et al. 1977); another was the relatively reduced value of acetoacetyl-CoA as substrates (Bennett et al. 1984). Hence, the diagnosis of CT-deficiency is not seemed practically easy. Furthermore, their diagnosis was not perfectly proven, because they were based on only the enzyme activities. Unfortunately, cells from these two patients could not be available for our method, because of extinction, although Drs. de Groot and Bennett, who reported these cases previously, kindly made efforts.

CT is involved in the biosynthesis of cholesterol or steroid hormones, catalyzing acetoacetyl-CoA from acetyl-CoA for substrate conversion into 3-hydroxy-3-methylglutaryl-CoA in the cytosol (Clinkenbeard et al. 1973). Recently, human CT cDNA was cloned and sequenced (Song et al. 1994) and assigned the gene to chromosome 6q25.3-q26 (Masuno et al. 1996). Accurate identification of CT-deficient patients may also be helpful in characterization or understandings of function of CT in tissues.

The method that we report here is simple and practical, and can make feasible early detection or intervention of CT-deficiency as well as other ketone body disorders, such as T2- or SCOT deficiencies. Furthermore, blood samples can be obtained less invasively. EB-lymphoblasts can grow more expansively after establishment of the cell lines, but the establishment may require a relatively long period, often 4 weeks or more. On the other hand, IL-2 lymphocytes proliferate more rapidly and may have advantages over EB-lymphoblasts for rapid diagnosis (Adolph et al. 1988), although the passage number of the culture is limited. Early detection of CT-deficient patients will be important to prevent neurological sequellae after the attacks or even to clarify the clinical concept of CT-deficiency.
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