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# Simple and unique purification by size-exclusion chromatography for an oligomeric enzyme, rat liver cytosolic acetyl-coenzyme A hydrolase

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**Abstract**

An overview of the purification of an oligomeric enzyme, an extramitochondrial acetyl-coenzyme A hydrolase from rat liver, is presented. The enzyme has been purified to homogeneity using two successive size-exclusion chromatography runs, first for the monomeric and second for the oligomeric form of the enzyme. The sequential gel-filtration steps efficiently removed the contaminants of any molecular size, first of different size from that of the monomeric form of the enzyme ($K_{av}$=0.47 on Superdex 200) and second of different size from that of the oligomeric form ($K_{av}$=0.33), allowing us to purify the enzyme in high purity. This strategy provides an excellent model for purifying many other oligomeric proteins including key enzymes or allosteric enzymes regulating metabolism.


*Keywords:* Oligomeric enzymes; Cystolic acetyl-coenzyme A hydrolase

## 1. Introduction

This review covers original papers on the purification of a cytosolic acetyl-coenzyme A (CoA) hydrolase (CACH, EC 3.1.2.1), which produces acetate and CoA from acetyl-CoA. The enzyme had escaped previous detection presumably due to its extreme cold lability until its first identification in rat liver in 1980 [1]. At room temperature the enzyme is usually present as active oligomeric forms, but at low temperature, they dissociate into an inactive monomer [2,3]. Because of its extreme cold lability, the enzyme had to be purified at room temperature (RT) [1–12] that caused a great loss of the enzyme activity, presumably due to denaturation of the enzyme protein by oxidation and degradation by contaminating proteases. Recently, the cold-inactivated monomeric form of the enzyme was found to be reconstituted to an active oligomeric form at 37 °C in the presence of a very low concentration of Triton X-100 [13]. The new finding allowed us to purify the cold-labile enzyme in the cold environment (0–4 °C) and further to develop an excellent purifying strategy using successive gel-filtration steps targeted for different quaternary structures of the enzyme.

## 2. Oligomeric properties of CACH

CACH had been detected in rat liver (~20 μmol/min per g wet mass of liver at 30 °C) and kidney

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(5% of the activity in liver cytosol per mg protein) [1,2]. We purified the enzyme [1] and demonstrated that at RT it is usually present as active forms: homodimer ($M_r$ 135 000) and homotetramer ($M_r$ 240 000) whose $K_m$ values for acetyl-CoA are 170 μ*M* and 60 μ*M*, respectively [2,3]. The active oligomeric forms are stable at RT or in 1.3 *M* sucrose, but at 0 °C in 0.3 *M* sucrose, they rapidly dissociate into an inactive monomer ($M_r$ 63 000) with a $t_{1/2}$ value of 3 min [4]. It is suggested that high sucrose concentrations prevent a very rapid dissociation process that occurs under isotonic conditions. The high rate of activity loss under the usual conditions of homogenization appears to explain the failure to detect activity previously. Allosteric effectors such as ATP (activator) and ADP (inhibitor) also regulated the enzyme activity [1]. Furthermore, these nucleotides cause association of the purified dimeric enzyme to a tetrameric form at high enzyme-protein concentrations at 25–37 °C [4], and only partially protect the dimer and tetramer against cold inactivation [4]. The phenomenon of cold lability is commonly found with oligomeric enzymes including allosteric enzymes regulating metabolism. At low temperature and low concentrations, these enzymes undergo a reversible loss of activity concomitant with dissociation into their corresponding subunits. Although it is generally thought that hydrophobic bonds [14] and ionization of groups of the enzyme molecules [15] are responsible for the effect of cooling, the phenomenon of cold lability is still enigmatic. Thus, the cold-labile and oligomeric CACH provides an excellent model system for studying many other oligomeric key enzymes.

## 3. Reassociation of inactive monomeric CACH to active oligomers

### *3.1. Effect of nucleotides, acetyl-CoA, phosphate, pyrophosphate, proteins, peptides or sucrose on rewarming*

It was found, with the purified CACH, that rewarming under appropriate conditions partially reversed the cold-induced inactivation and dissociation of the enzyme: at a low protein concentration of 14 μg/ml, simple rewarming only partially restored the enzyme activity (less than 3% of the original activity), while at a higher concentration of 370 μg/ml, the reactivation by warming was greater (about 90% of the initial enzyme activity) [3]. Warming at 37 °C appeared to be optimal for reactivation; warming at 25 °C or at 43 °C was less effective [3]. Among various nucleotides tested, ATP greatly enhanced the restoration of the activity, while ITP, UTP and ADP were less effective and AMP, GTP, TTP and CTP had little effect [3]. At a low enzyme-protein concentration of 14 μg/ml, 2 m*M* ATP restored the enzyme activity to about 70% of that before cold treatment, while acetyl-CoA (0.5 m*M*) restored the activity about 50% [3]. High concentrations of phosphate (0.92 *M*) and pyrophosphate (0.45 *M*) restored about 80 and 95%, respectively, of the original activity [3]. Among various proteins or peptides tested, 1 mg/ml bovine serum albumin (BSA) greatly enhanced the reactivation of the cold-inactivated enzyme (about 80%), bovine IgG (1 mg/ml) was less effective, and human adrenocorticotropic hormone (0.33 mg/ml) had no effect, while human insulin (0.08–0.33 mg/ml) inhibited the enzyme reactivation in a dose-dependent manner [16]. Although various molecules restored the activity appreciably, they were not effective at low temperature. Thus the reactivation and reassociation of enzyme were absolutely dependent on the temperature. A high concentration of sucrose (≥1.3 *M*) did not enhance the conversion of the monomeric enzyme to the oligomeric active forms on rewarming [3]. This may be because the high viscosity of sucrose reduced the ease of aggregation of molecules or changed the interaction between the protein and water. The physicochemical mechanism of the process of reversible activation or association of this enzyme has yet to be solved.

### *3.2. Effect of low concentration of Triton X-100*

Recently, non-ionic detergent Triton X-100 has been revealed to be an excellent reactivator of the cold-inactivated CACH. In all of diluted CACH samples tested (ranging in enzyme-protein concentration from 1.8 to 7.2 μg/ml), the enzyme could be almost fully reactivated with 0.01% (0.16 m*M*) Triton X-100 (Fig. 1A). The time course of restoration of the enzyme activity is also shown in Fig. 1A;

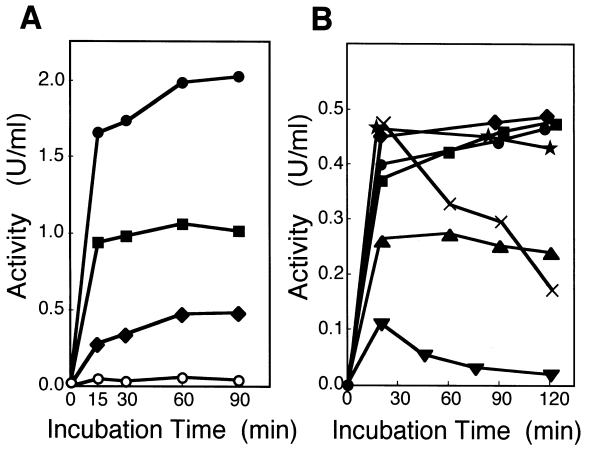


Fig. 1. Time course of reactivation of acetyl-CoA hydrolase. The cold-inactivated enzymes were reactivated by warming (described in the text) in the presence of Triton X-100. Data represent the mean of three separate experiments. (A) Reactivation in the presence of 0.16 m*M* Triton X-100 at protein concentrations of 1.8 μg (0.5 U)/ml (♦), 3.6 μg (1.0 U)/ml (■), 7.2 μg (2.0 U)/ml (●), and in the absence of Triton X-100 at a protein concentration of 7.2 μg/ml (○). (B) Reactivation in a protein concentration (1.8 μg/ml) in the presence of various concentrations of Triton X-100: $2.2\times10^{-7}$ *M* (▼), $1.6\times10^{-6}$ *M* (▲), $1.6\times10^{-5}$ *M* (■), $1.6\times10^{-4}$ *M* (●), $1.6\times10^{-3}$ *M* (♦), $1.6\times10^{-2}$ *M* (★), and $5.1\times10^{-2}$ *M* (×).

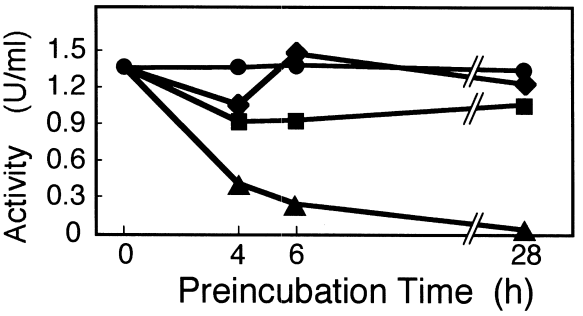


Fig. 2. Dose-dependent effects of Triton X-100 on the stabilization of acetyl-CoA hydrolase. The enzyme was preincubated at 4 °C in a protein concentration (5.4 μg/ml) in the presence of various concentrations of Triton X-100: $6.4\times10^{-6}$ *M* (▲), $1.6\times10^{-5}$ *M* (■), $1.6\times10^{-4}$ *M* (●) and $1.6\times10^{-3}$ *M* (♦). After the preincubation for an indicated period, the enzyme was then reactivated by warming (described in the text) and the remaining activity was measured. Data represent the mean of three separate experiments.

the activity was rapidly increased by warming and reached a plateau in 60 min and remained at this level until 90 min. At an enzyme-protein concentration of 1.8 μg/ml, the enzyme activity was restored almost fully by a wide concentration range of Triton X-100 ($1.6\times10^{-5}$–$5.1\times10^{-2}$ *M*) (Fig. 1B). At the highest concentration tested ($5.1\times10^{-2}$ *M*), however, the restoration was followed by rapid loss of the activity (Fig. 1B).

As we reported previously [3], the enzyme in a highly diluted sample (14 μg/ml) was irreversibly inactivated at 4 °C even in the presence of a stabilizer (BSA). We have recently found that even the highly diluted enzyme (5.4 μg/ml) escaped from denaturation at 4 °C by the low concentration of Triton X-100, which had been known as a good stabilizer for a cytosolic phospholipase $A_2$ of bovine adrenal medulla besides the membranous proteins [17]. The detergent (range $1.6\times10^{-5}$–$1.6\times10^{-3}$ *M*) protected the diluted CACH over 28 h on an ice bath against irreversible loss of activity (Fig. 2). However, a very low concentration ($6.4\times10^{-6}$ *M*) of the detergent did not show a protective effect on the enzyme (Fig. 2). Triton X-100 stabilized and reactivated the enzyme maximally at a very low concentration (0.016 m*M*) (Figs. 1B and 2), a level far below the critical micellar concentration (0.3 m*M*) [18]. Thus, single molecules, but not the micellar form, are sufficient for the Triton X-100 actions on the enzyme [19]. In the monomeric form of Triton X-100, both of its hydrophobic and hydrophilic moieties may interact with the enzyme, in contrast to the micellar form that has only the polar moieties on the surface [19]. Moreover, because the Triton X-100 actions were still observed in the presence of high concentrations of salt (1 *M* potassium phosphate) (Fig. 1), the hydrophobic moieties of Triton X-100 may act on cytosolic acetyl-CoA hydrolase. High concentrations of hydrophilic substances (salt and polyols) strongly protect the dimeric acetyl-CoA hydrolase against dissociation into inactive monomer [7], suggesting that hydrophobic interactions play an important role in the protein–protein interaction. Presumably, Triton X-100 may interact with hydrophobic surfaces of acetyl-CoA hydrolase protein, which are prerequisite for self-assembly. The stabilization of hydrophobic surfaces of the monomeric enzyme by Triton X-100 may protect the monomeric enzyme against denaturation and also promote its oligomerization. Alternatively, it is also possible to

explain in terms of Timasheff's protein–solvent general thermodynamically unfavorable interaction [20–22], without the need to invoke any specific interaction between protein and the solvent systems. The actual mechanism of interaction between the enzyme and Triton X-100 is still an open question. However, the definitive answers to the question have to await the X-ray crystallographic or nuclear magnetic resonance spectrometric (NMR) studies on the CACH structure, and the structural characterization should help in the elucidation of mechanisms controlling its catalytic activity based on the association equilibria.

## 4. Novel purification method of the cold-labile CACH in the cold environment

With the aid of 0.01% Triton X-100, an effective stabilizer as well as a reactivator of the cold-inactivated CACH, we have developed a novel purification method of cold-labile CACH in the cold environment. The reconstitution of the monomeric to oligomeric form of the enzyme in the presence of Triton X-100 further allowed us to adopt an excellent purifying strategy using two successive size-exclusion chromatography steps, first for the monomeric and second for the oligomeric form of the enzyme.

### *4.1. Materials*

Male albino rats (200–250 g), *Rattus norvegicus* strain Donryu, were given a single subcutaneous injection of 50 mg/100 g body weight of 2-(*p*-chlorophenoxy)isobutyric acid (CPIB), a hypolipidemic drug, for CACH induction [3,5]. The drug-treated rats were sacrificed by decapitation under ether anesthesia on day 2 after the injection, when we have the highest CACH activity in whole liver [5].

### *4.2. Purification at 4 °C—using temperature-sensitive quaternary conformational change in the enzyme protein*

All steps were carried out at 4 °C, except for the last gel filtration at 25 °C. For the enzyme assay of cold-inactivated CACH samples from steps 1–3, an aliquot of each sample was mixed with 2 volumes of buffer A: 1.5 *M* $KH_2PO_4$/KOH (pH 7.2), 250 m*M* sucrose, 2 m*M* dithiothreitol, 0.5 m*M* phenylmethylsulfonyl fluoride and 0.02% $NaN_3$. The mixture was then incubated at 37 °C for 60 min to reactivate the enzyme. For the assay of cold-inactivated CACH samples containing 0.01% Triton X-100 from step 4, an aliquot of each sample was incubated at 37 °C for 30 min to reactivate the enzyme. Protein content was determined using a Bio-Rad protein assay kit based on the method of Bradford [23] using BSA as a standard, or in the cases of the Mono Q and the Superdex 200 fractions by ultraviolet absorption.

#### *4.2.1. Step 1: preparation of cytosolic fraction*

The livers (152 g) from the CPIB-treated rats were perfused with buffer B: 25 m*M* $KH_2PO_4$/KOH (pH 7.2), 250 m*M* sucrose, 2 m*M* dithiothreitol, 0.5 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride and 0.02% $NaN_3$. They were excised and then homogenized 1:2 (v/v) in buffer B. The homogenate was centrifuged at 20 000 *g* for 15 min and the resultant supernatant was further centrifuged at 113 000 *g* for 1 h to obtain a cytosolic fraction.

#### *4.2.2. Step 2: DEAE-cellulose column chromatography*

The cytosolic fraction was loaded onto a DEAE-cellulose (DE-52, Whatman) column (94 mm×26 mm) equilibrated with buffer B. The column was washed and the enzyme was eluted with buffer B containing 0.15 *M* NaCl.

#### *4.2.3. Step 3: Mono Q FPLC*

The sample from the Step 2 was desalted with a PD-10 column (Amersham Pharmacia) and then applied to a fast protein liquid chromatography (FPLC) Mono Q column (HR 10/10, Amersham Pharmacia) equilibrated with buffer C: 25 m*M* $KH_2PO_4$/KOH (pH 7.2), 2 m*M* dithiothreitol, 0.5 m*M* EDTA, 0.5 m*M* phenylmethylsulfonyl fluoride and 0.02% $NaN_3$. The column was developed with a linear 0–0.5 *M* KCl gradient in 2.7 ml buffer C. Active fractions were pooled and concentrated using a Centricon-30 (Millipore) $M_r$ 30 000 cut-off microconcentrator. The concentrated sample was mixed with Triton X-100 (0.16 m*M*) and then incubated at

4 °C overnight. The preparation was then clarified by centrifugation at 15 000 *g* for 15 min.

#### *4.2.4. Step 4: first gel filtration at 4 °C for purifying monomeric form of the enzyme*

The clarified sample was loaded onto a Superdex 200 column (PC 3.2/30) on a Smart HPLC System (Amersham Pharmacia) equilibrated with buffer D: 25 m*M* $KH_2PO_4$/KOH (pH 7.2), 150 m*M* KCl, 2 m*M* dithiothreitol and 0.16 m*M* (0.01%) Triton X-100. The monomeric enzyme was eluted at a rate of 40 μl $min^{-1}$ at 4 °C. Fractions containing the activity were pooled, concentrated using a Nanosep-10K (Pall Filtron) microconcentrator and then incubated at 37 °C for 60 min to convert the monomeric enzyme to the active oligomer.

#### *4.2.5. Step 5: second gel filtration at 25 °C for purifying oligomeric form of the enzyme*

Finally, the oligomeric enzyme sample was subjected to the Superdex 200 column again as in Step 4 except carried out at 25 °C. The active oligomeric form was maintained during this step. Fractions containing the activity were pooled and stored frozen at −80 °C until use.

### *4.3. Advantages*

In the novel purification method [13], CACH was purified to homogeneity in a high yield (about fourfold) compared with that of our previous method [1]. In the present method, the livers of the CPIB-treated rats were used [13]. CPIB treatment doubled the enzyme activity in rat liver [4] and thus minimized the sacrificed rats to about one-third [1,2]. As shown in Fig. 3, the cold-inactivated monomeric form of the purified enzyme ($M_r$ 62 000 estimated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS/PAGE)) was eluted from the Superdex 200 column at 4 °C unexpectedly at $M_r \approx 37\,000$ ($K_{av}=0.47$), suggesting some interactions between the monomeric enzyme protein and the gel matrix. After the reactivation of the enzyme sample at 37 °C in the presence of 0.01% Triton X-100, the activity was then eluted at $M_r \approx 124\,000$ ($K_{av}=0.33$) from the same column at 25 °C, demonstrating that the reactivation procedure resulted in oligomerization of the monomeric enzyme. The alternate gel-filtration steps,

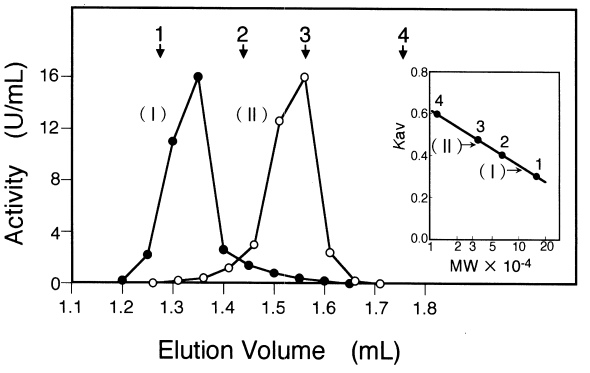


Fig. 3. Elution profiles of purified rat cytosolic acetyl-CoA hydrolase on gel filtration. The purified CACH samples, either in the reactivated (I) or cold-inactivated (II) form, were applied to Superdex 200 PC 3.2/30 and eluted at a rate of 40 μl $min^{-1}$ at 25 °C (●) or at 4 °C (○), respectively. Assays of the enzyme activity were done immediately after elution for active samples (I) or after further reactivation procedure for inactive samples (II). The molecular mass markers are as follows: 1, IgG (160 000); 2, BSA (67 000); 3, β-lactoglobulin (35 000); 4, cytochrome *c* (12 400). The molecular mass was estimated by interpolation from a standard plot of the molecular mass against $K_{av}$ (inset). $K_{av}=(V_e-V_o)/(V_t-V_o)$ where $V_e$ is elution volume, $V_o$ is void volume, and $V_t$ is column volume.

Step 4 for the dissociated form with the low apparent molecular mass and Step 5 for the associated form of CACH, efficiently removed the contaminants of any molecular size, resulting in a high purity with high recovery (72%, see Table 1, from Step 4 to Step 5).

## 5. Conclusions

We previously demonstrated that CACH is an allosteric enzyme regulated by ATP (activator) and ADP (inhibitor) [1,2]. Physiological observations in rat liver have revealed that CACH activity greatly increases in opposite metabolic states: in enhanced fatty acid oxidation and in heightened fatty acid synthesis [1,4]. Further, this enzyme was markedly induced by CPIB [5], a hypolipidemic drug or a peroxisome proliferator, which enhances rat liver mitochondrial and peroxisomal β-oxidation [24] and increases cytosolic CoA level [25,26]. Thus, CACH is presumably a key enzyme involved in fat metabolism and its physiological role has been postulated to

Table 1
Purification scheme for the cytosolic acetyl-CoA hydrolase from rat liver

| Step | Total activity (U) | Total protein (mg) | Specific activity (U mg$^{-1}$) | Purification (*x*-fold) | Yield (%) |
|---|---|---|---|---|---|
| (1) Cytosol | 2155 | 5360 | 0.40 | – | 100.0 |
| (2) DE-52 | 947 | 1894 | 0.50 | 1.3 | 43.9 |
| (3) Mono Q | 284 | 59.2 | 4.8 | 12.0 | 13.2 |
| (4) Superdex 200 | 232 | 15.8 | 14.7 | 36.8 | 10.8 |
| (5) Superdex 200 | 204 | 0.62 | 329 | 823.0 | 9.5 |

The cytosolic acetyl-CoA hydrolase was purified from the livers of 10 male rats treated with CPIB 2 days before. Livers were homogenized and the homogenate was centrifuged to yield a cytosolic fraction (cytosol) as described in the text (step 1; Section 4.2.1). Triton X-100 was added to the buffer used for Superdex-200 chromatography to a final concentration of 0.16 m*M* to stabilize the enzyme. All steps were carried out at 4 °C, except for the last one at 25 °C. For the assay of cold-inactivated CACH samples, they were reactivated by warming as described in the text.

be to supply cytosolic free CoA for both fatty acid synthesis and oxidation [4].

The enzyme had escaped previous detection presumably due to its extreme cold inactivation associated with conformational change in its quaternary structure. The phenomenon entails widespread oligomeric enzymes known as allosteric key enzymes, and has been subject to hindrance for their purification. We presented here the reactivation procedure for the cold-inactivated monomeric CACH, allowing us to purify the cold-labile enzyme in the cold environment (0–4 °C) in a high yield, and further in high purity using successive gel-filtration targeted for different quaternary structures of the enzyme. The present method provides an excellent model for purifying many other oligomeric proteins such as metabolic key enzymes.

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