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Purification and Properties of Isocitrate Lyase from Pupas of the Butterfly *Papilio machaon* L.

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Abstract—Key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, were identified in pupas of the butterfly *Papilio machaon* L. The activities of these enzymes in pupas were 0.056 and 0.108 unit per mg protein, respectively. Isocitrate lyase was purified by a combination of various chromatographic steps including ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Toyopearl, and gel filtration. The specific activity of the purified enzyme was 5.5 units per mg protein, which corresponded to 98-fold purification and 6% yield. The enzyme followed Michaelis–Menten kinetics (K_m for isocitrate, 1.4 mM) and was competitively inhibited by succinate (K_i = 1.8 mM) and malate (K_i = 1 mM). The study of physicochemical properties of the enzyme showed that it is a homodimer with a subunit molecular weight of 68 ± 2 kD and a pH optimum of 7.5 (in Tris-HCl buffer).

Key words: glyoxylate cycle, isocitrate lyase, malate synthase, kinetic characteristics, diapause

It is generally believed that the key enzymes of the glyoxylate cycle, isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2), do not occur in animal tissues and function solely in microorganisms and higher plants [1]. Nevertheless, ICL and MS activities were detected at larval ontogeny stages and embryogenesis of some nematodes [2]. For example, it was shown that the mitochondrial fraction of *Ascaris suum* larvae contained ICL and MS, which ensure the synthesis of glycogen from fatty acids [3]. In addition, some biochemical and cytochemical studies showed that the key enzymes of the glyoxylate cycle are present in tissues of higher animals. The activities of ICL and MS were detected in renal tubules of *Bufo marinus* [4] and in the liver of guinea pig [5] and chicken [6]. Note that the expression of these enzymes in tissues of higher animals is usually observed under extreme or pathological conditions [7]. For example, the key enzymes of the glyoxylate cycle were found in peroxisomes of hepatocytes of starving rats and rats suffering from alloxan diabetes [8-10]. The glyoxylate cycle ensures the conversion of two-carbon acetyl-CoA into a four-carbon glucogenic substrate, succinate, thereby relating lipolysis and gluconeogenesis.

Fats play a pivotal role in metabolism of insects because they are one of the main sources that yield energy during molting, metamorphoses, imaginal aphagia, and starving. It was assumed that trehalose, found in many insects, performs the same function as glucose in blood of mammals [11]. The importance of gluconeogenetic transformation for this group of invertebrates is determined by the necessity of maintenance of the physiological concentration of trehalose, which plays a key role in metabolism of insects [12]. Unfortunately, considering that carbohydrate metabolism of insects is poorly studied, the enzymatic organization of gluconeogenesis in inserts remains obscure [13]. To date, it has remained unclear whether the glyoxylate cycle indeed plays an important role in metabolism of insects because data on its distribution in insects are absent.

The purpose of this work was to detect the activities of the key enzymes of the glyoxylate cycle in pupas of the butterfly *Papilio machaon* L. and to obtain an electrophoretically homogeneous preparation of ICL for studying its physicochemical, catalytic, and regulatory properties.

MATERIALS AND METHODS

This study was performed with the butterfly *Papilio machaon* L. Fertilized females, captured in nature, were placed into warrens for ovipositing. The butterflies were fed with 5% sucrose. Eggs together with the forage plant were transferred onto Petri dishes (approximately 100 eggs per dish). Caterpillars were then grown in 1.5-dm^3 vessels

Abbreviations: ICL) isocitrate lyase; MS) malate synthase.

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(density, five to seven caterpillars per vessel). The caterpillars that stopped feeding were then placed into special warrens for pupation. The pupas were stored at 4°C. Diapause of dormant pupas was interrupted by incubating them at 25°C, which triggered their metamorphosis to convert to imagoes [14]. Pupas (1 g) were homogenized in a medium (5 ml) containing 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 5 mM DTT, and 3 mM EDTA. Debris was precipitated by centrifugation at 3000*g* for 5 min.

The activity of ICL was determined spectrophotometrically by the method of Dixon at 324 nm [15]. The ration medium contained 50 mM Tris-HCl (pH 7.5), 5 mM isocitrate, 3 mM $MgCl_2$, and 4 mM phenylhydrazine. The activity of MS was monitored at 412 nm by the formation of the complex between CoA and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), as described in [16]. The reaction medium contained 50 mM Tris-HCl (pH 7.5), 150 μM acetyl-CoA, 1 mM DTNB, and 2 mM glyoxylate. The amount of enzyme that transformed 1 μmol of substrate in 1 min at 25°C and optimal pH was taken as one unit of activity. The content of protein in the sample was determined by the method of Lowry et al. [17].

Isocitrate lyase from *P. machaon* pupas was purified according to the following scheme developed by us: 1) fractionation with ammonium sulfate (30-65% saturation); 2) desalting of the protein preparation by gel filtration on a column (15 × 1.7 cm) packed with Sephadex G-25 (Pharmacia, Sweden), equilibrated in 10 mM Tris-HCl (pH 7.5) containing 1 mM DTT and 1 mM $MgCl_2$; 3) ion-exchange chromatography on a column (20 × 1.8 cm) packed with DEAE-Toyopearl, equilibrated in 10 mM Tris-HCl (pH 7.5), and subsequent elution with a KCl linear gradient; 4) gel filtration on a column (80 × 2 cm) with Toyopearl HW-65 (Toyo-Soda, Japan), equilibrated in buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM DTT, 1 mM EDTA, and 3 mM $MgCl_2$. All procedures were performed at 0-4°C.

Electrophoresis was performed by the method of Davis with some modifications, as described in [18], in 8% polyacrylamide gel. Nonspecific staining of gels was performed using silver nitrate [19]. For specific development, gels were placed in medium (20 ml) containing 50 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 3 mM $MgCl_2$, 3 mM DTT, 10 mM isocitrate, and 1.2 ml of modified Schiff's reagent [20]. The control samples did not contain isocitrate and $MgCl_2$. Gels were incubated at 37°C until a bright red band appeared. Preparations were analyzed by SDS-PAGE using the detergent system suggested by Laemmli [21]. Before loading onto gels, the samples containing 5% β-mercaptoethanol, 10% glycerol, and 10% SDS were heated in a water bath (90°C) for 10 min. The molecular weight of subunits was determined using the marker proteins cellulase (94.6 kD), BSA (66.2 kD), ovalbumin (45 kD), carboanhydrase (31 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). The gels were stored in 7% acetic acid.

The molecular weight of the enzyme was determined by gel chromatography on Toyopearl HW-65, using a calibration curve plotted using the molecular weights of the marker proteins catalase (120 kD), BSA (66.2 kD), egg albumin (45 kD), and peroxidase (45 kD).

The results shown in the table and figures are the mean values and standard deviations. The number of experiments is shown in parentheses.

RESULTS

The activities of the marker enzymes of the glyoxylate cycle, ICL and MS, were detected in pupas of the butterfly *P. machaon* in diapause (0.023 and 0.044 unit per mg protein, respectively). More than a twofold increase in ICL and MS activities (0.056 and 0.108 unit per mg protein, respectively) was observed in the *P. machaon* pupas that were incubated at 25°C for 7 days (the emergence of imagoes occurred on day 8 or 9).

To study physicochemical and regulatory properties of ICL, we purified this enzyme from reactivated pupas. The results of a typical purification procedure are summarized in the table.

Purification of isocitrate lyase from pupas of the butterfly *P. machaon* L.

Purification stage	Volume, ml	Total activity, units	Protein content, mg	Specific activity, units/mg	Yield, %	Degree of purification
Homogenate	12.6	5.3	94.05	0.06	100	1.0
Fractionation with $(NH_4)_2SO_4$	1.8	4.9	40.95	0.12	93	2.1
Gel filtration on Sephadex G-25	5.1	4.8	35.64	0.13	91	2.4
Ion-exchange chromatography on DEAE-Toyopearl	6.0	1.2	0.70	1.76	23	31.4
Gel filtration of Toyopearl HW-65	6.5	0.4	0.08	5.54	6	98.2

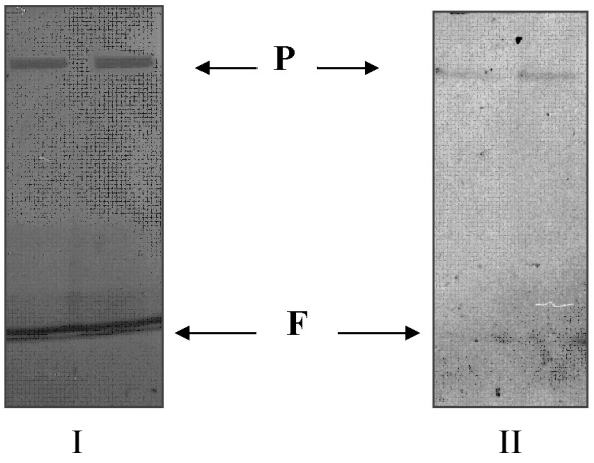

Fig. 1. Electrophoregram of isocitrate lyase purified from pupas of the butterfly *P. machaon* L.: I) staining with silver nitrate; II) specific development; P) protein band; F) bromophenol blue front.

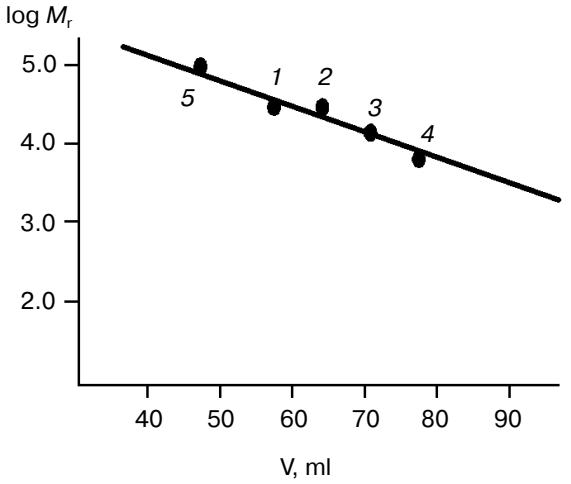

Fig. 2. Determination of molecular weight of native isocitrate lyase from *P. machaon* pupas ($n = 6$) by gel chromatography on Toyopearl HW-65: *1*) catalase; *2*) BSA; *3*) egg albumin; *4*) peroxidase; *5*) ICL.

As a result of successively performed fractionation with ammonium sulfate, gel filtration on Sephadex G-25, ion-exchange chromatography on DEAE-Toyopearl, and gel chromatography on Toyopearl HW-65, we obtained enzyme preparation with a specific activity of 5.5 units per mg protein, which corresponded to 98-fold purification and 6% yield.

The analysis of the preparation by PAGE under nondenaturing conditions showed the presence of a single protein band with $R_f = 0.24$. Specific staining of gels for ICL showed that the only protein band corresponded to the activity of ICL (Fig. 1).

In the course of our studies, we selected the conditions that ensured long-term storage of ICL. The activity of homogeneous preparation of ICL, stored at 4°C, decreased by 10 to 15% during the first day and then by 10% during each day of storage. Optimal storage medium for ICL contained 0.1 M Tris-HCl (pH 7.5), 3 mM β-mercaptoethanol or 6 mM DTT, 5 mM $MgCl_2$, and 25% glycerol. In this case, 90% of initial activity was retained during first 5 days; 25%, after 30 days of storage.

The molecular weight of ICL from *P. machaon* pupas, determined by gel filtration on Toyopearl HW-65, was 138 ± 3 kD (Fig. 2). SDS-PAGE analysis showed that the molecular weight of ICL subunits was 68 ± 3 kD (Fig. 3). The study of kinetic and regulatory properties of ICL showed that the enzyme obeyed Michaelis–Menten kinetics (K_m for isocitrate, 1.4 mM). To study pH optimum of this enzyme, we used different buffers (Tris-HCl, HEPES-KOH, and NaH_2PO_4-Na_2HPO_4). The greatest activity of ICL was observed in Tris-HCl (pH 7.5).

When studying the effect of metabolic intermediates on the activity of ICL from *P. machaon* pupas, we found that malate and succinate inhibited the enzyme in a competitive mode. Inhibition constant (K_i), calculated by the method of Dixon [22] for glucose-6-phosphate, was 0.3 mM; fructose-6-phosphate, 60 μM; succinate, 1.8 mM; and malate, 1 mM.

Incubation of ICL with amino acids (serine, glycine, and aspartic and glutamic acids) did not cause significant changes in enzymatic activity. Similarly, the incubation of ICL with divalent cations (Co^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+}, and Cd^{2+}) at concentrations of 5, 15, and 30 mM for 15, 30, and 60 min did not change its activity. The ions Na^+, K^+, and Cl^- also had no effect on the ICL activity.

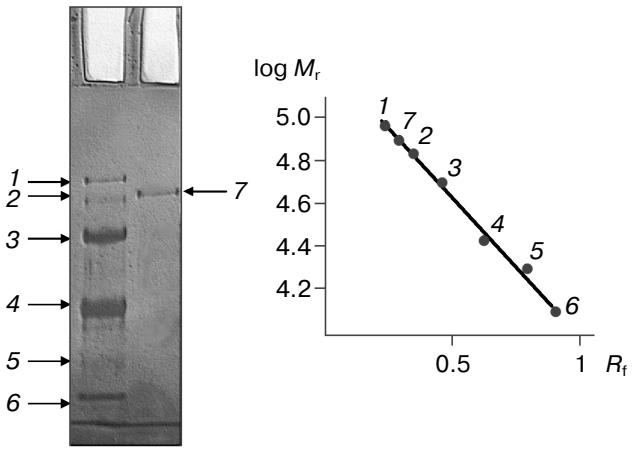

Fig. 3. Electrophoregram and calibration curve for the determination of subunit molecular weight of isocitrate lyase from *P. machaon* pupas by SDS-PAGE: *1*) cellulase (94.6 kD); *2*) BSA (66.2 kD); *3*) ovalbumin (45 kD); *4*) carboanhydrase (31 kD); *5*) trypsin inhibitor (21.5 kD); *6*) lysozyme (14.4 kD); *7*) ICL.

DISCUSSION

Due to relative chemical inertness, neutral fats can be accumulated in insects in large amounts without affecting other biochemical processes. Fats serve as one of the main energy-yielding sources during marginal aphagia, starvation, molting, metamorphoses, oogenesis, and sex activity [11]. The process of metamorphosis in insects is related to rearrangement of organs (in particular, to the synthesis of chitin skeleton). The degradation products of pupal tissues and glycogen stores may be used for the synthesis of chitin; however, the main source of carbon during metamorphosis is the fat body, which contains neutral triacylglycerols. In plants and microorganisms, gluconeogenesis from lipids is ensured by peroxisomal or mitochondrial β-oxidation of fatty acids, glyoxylate cycle, a section of Krebs cycle, phosphoenolpyruvate carboxykinase, and reversed glycolysis [8].

The question on the function of the glyoxylate cycle in animal tissues remains open. This process has been described for nematodes and trematodes, amphibians, birds, and mammals. The activities of ICL and MS have been detected in rat liver and kidneys on food deprivation and experimental diabetes; in chick liver, after injecting vitamin D to birds deficient in this vitamin (i.e., under the conditions of mobilization of reserve lipids to replenish the pool of carbohydrates) [6]. In this study, we detected the activities of the key enzymes of the glyoxylate cycle (ICL and MS) in insects, which suggests that this cycle may be involved in the conversion of fatty acids into carbohydrates.

The activities of these enzymes in *P. machaon* pupas are comparable to the activities in plant [23, 24], fungal [25], and mammalian [9] ICL and MS. The use of a five-stage purification procedure made it possible for the first time to obtain a homogeneous preparation of the ICL from *P. machaon* pupas (specific activity, 5.5 units per mg protein). To date, only ICLs from several species of microorganisms [26], plants [24], nematodes [27], ticks [28], and rats [7] have been purified to homogeneity. Dimeric structure, characteristic of ICL from *P. machaon*, was observed earlier only in the ICL from the yeast *Candida tropicalis*, whose subunits have a molecular weight of 65 kD [22]. A similar subunit molecular weight has been reported for ICLs from the nematode *Caenorhabditis elegans* (61.6 kD) [29], *Lupinus* seeds (66 kD) [23], and *Pinus densiflora* pollen (65 kD) [24]. However, in the first two cases, ICL is a tetramer, and in the last case, it has a trimeric structure. The analysis of the results of ion-exchange chromatography (the enzyme was eluted with 70 mM KCl as a single peak) and the pattern of specific development of ICL after PAGE (a band with $R_f = 0.24$) led us to the conclusion that only one ICL isoform functions in the *P. machaon* pupas. Interestingly, five isoforms of this enzyme were detected in the nematode *Turbatrix aceti* [27]; two isoforms, in the bacteria *Pseudomonas* sp. [26]; and one isoform, in the liver of starving rat [9].

The inhibition of ICL by a trehalose precursor, glucose-6-phosphate, additionally confirms the assumption that the physiological role of the glyoxylate cycle is the utilization of fatty acids to maintain sufficient level of carbohydrates and energy balance in cells.

The results of the study of physicochemical and regulatory properties of ICL from *P. machaon* pupas suggest that the glyoxylate cycle probably functions in insects and, therefore, is more widespread than it has been believed earlier.

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