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Physicochemical and Kinetic Characteristics of Isoforms of Isocitrate Lyase from Corn

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Abstract—Three electrophoretically homogeneous isocitrate lyase (ICL) isoforms were obtained by 4-step purification from corn scutellum (ICL_1 and ICL_2) and green leaves (ICL). Their physicochemical, kinetic, and regulatory properties were analyzed. The molecular masses of ICL_1, ICL_2, and ICL isoforms determined by gel filtration are 164, 207, and 208 kDa, respectively. The proteins have homotetrameric quaternary structure with subunit molecular masses of 43, 48, and 47 kDa for ICL_1, ICL_2, and ICL, respectively. We found some differences in pH optimum, K_m, and regulation by divalent metal cations between ICL_1 and ICL_2 and significant similarity of ICL_2 and ICL. Based on these data, we suggest the participation of these isoforms in metabolic regulation of the glyoxylate cycle, organic acid metabolism during photorespiration in leaves and acidosis in corn seeds.

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Key words: isocitrate lyase, isoforms, electrophoresis, quaternary structure, subunits, glyoxylate cycle, gluconeogenesis

Isocitrate lyase (ICL; EC 4.1.3.1) is one of the key enzymes of the glyoxylate cycle (GC) that functions in different physiological periods of life of many organisms. This metabolic pathway was discovered in 1957 by Kornberg and Krebs in bacteria using acetate as a carbon source [1]. For plants, this enzyme is the key one in GC function at the stage of germination, providing the most important process: conversion of reserve lipids into carbohydrates in fat-storage seeds [2]. The activity of ICL usually correlates with the fat content in seeds [3]. However, germination is not the only physiological situation that leads to the induction of isocitrate lyase. The list of angiosperm plants known to have the GC is now rather extensive [4, 5]. The glyoxylate cycle is the most important mechanism of condensation of two acetyl-CoA molecules formed during utilization of lipids in aging leaves of plants into a glucogenic substrate (succinate) [6]. It is known that the activity of isocitrate lyase increases during plant aging, allowing the transformation of lipids into organic acids [7, 8]. The presence of isocitrate lyase has also been reported in tissue culture cells of rose petals and in green leaves of some plants, where its activity is camouflaged by endogenous inhibitors [9].

Although ICL is mainly a glyoxysomal enzyme involved essentially in the GC, there is information about the presence of a second enzyme form [10]. The analysis of genetic databases (GenBank, Swiss-Prot (http://www.ncbi.nlm.nih.gov)) has shown that the genome of *Arabidopsis thaliana* contains two ICL genes located on different chromosomes. The *icl1* gene is located in locus AT1G21440 of chromosome 1 and the *icl2* gene is located in locus AT3G21720 of chromosome 3. The previously revealed second extra-glyoxysomal isoform of the enzyme substantially differs from glyoxysomal isocitrate lyase of fat-storage tissues, is probably localized in the cytoplasm, and participates in utilization of glyoxylate formed during photorespiration [10]. However, only one ICL form providing GC functioning has been isolated from corn scutellum, which is the most metabolically active part of a seed [11].

The objectives of the present work were as follows: to study ICL polymorphism in corn seeds and green leaves, to obtain its isoenzymes in a homogenous state from different organs, and to determine their physicochemical and kinetic characteristics.

MATERIALS AND METHODS

The objects of research were scutella of 4-day-old etiolated corn seedlings (*Zea mays* L., variety Voronezhskaya 76) grown hydroponically at 25°C.

Abbreviations: DTT, dithiothreitol; GC, glyoxylate cycle; ICL, isocitrate lyase.

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The activity of isocitrate lyase was determined by spectrophotometry using an SF-46 instrument (LOMO, Russia) by the change in light absorption at 324 nm due to formation of a phenylhydrazine–glyoxylate complex [12]. The reaction medium contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 4 mM dithiothreitol (DTT), 2 mM sodium isocitrate, and 4 mM hydrochloric acid phenylhydrazine. The enzyme quantity providing the formation of 1 μmol glyoxylate in 1 min at 25°C was taken as a unit of enzyme activity. Total protein was assayed by the method of Lowry et al. [13].

High-purity isocitrate lyase preparations were obtained using a four-stage purification scheme. All procedures were performed at 0-4°C. A weighed portion of plant material (5 g) was homogenized in five volumes of the isolation medium (50 mM Tris-HCl buffer, pH 7.5, containing 3 mM EDTA, 0.1 M $MgCl_2$, and 5 mM DTT) and centrifuged for 5 min at 5000*g*. The supernatant proteins were fractionated by ammonium sulfate at saturation of 0-40%. The resulting solution was centrifuged for 20 min at 12,000*g*. Gel filtration was performed on a column (1.5 × 20 cm) with Sephadex G-25 (Pharmacia, Sweden). Proteins were eluted by 50 mM Tris-HCl buffer (pH 7.5) at a rate of 15-20 ml/h. Ion-exchange chromatography was performed in a column (1.5 × 1.5 cm) with DEAE-cellulose (Whatman, GB) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The enzyme was desorbed from the column by a linear gradient with KCl concentration of 50 to 150 mM in the same buffer.

The quaternary structure and molecular weight of native ICL were determined by gel chromatography on a column (2 × 40 cm) with Sephadex G-200 [14]. The volume of its yield (V_e) and the free volume (V_o) of the column with blue dextran (Serva, USA) were determined.

Electrophoretic analysis of the proteins was performed in 7.5% polyacrylamide gel [15]. Proteins were universally developed by silver nitrate. Gels were stored in 7% acetic acid solution. The enzyme was identified with a modified Schiff reagent [16]. SDS-PAGE was carried out in 12.5% polyacrylamide gel. Each sample contained 3-5 μg of protein. Calibration curve was plotted using standard marker proteins (Sigma, USA) [17].

Experiments were performed in 3-4 biological repeats; analytic definitions for each sample were made three times. The table and figures show the data from typical experiments, each value being the mean of three definitions. The data were treated using the Student *t*-criterion. The differences statistically reliable at $p < 0.05$ are discussed [18].

RESULTS AND DISCUSSION

Electrophoresis in 7.5% polyacrylamide gel followed by specific staining for ICL activity showed the presence of two enzyme isoforms with different electrophoretic mobility in corn scutella (Fig. 1). One form was revealed in green leaves, and its electrophoretic mobility value (0.25) coincided with the R_f value of one of the forms in the scutella. Multiple ICL forms have been described previously in pine and soya, where the enzyme is revealed as at least three different isoforms [19, 20].

The modified four-stage purification scheme provided two ICL isoforms from corn scutella in homogenous state. The results of typical purification are shown in the table. On elution of the proteins from DEAE-cellulose with a linear gradient of KCl (50-150 mM), the specific activity of ICL_1 was 4.9 U/mg. The purification degree was 123 and the yield was 5.7%. For isoform ICL_2, the specific activity was 4.4 U/mg and the purification degree and yield were 110 and 5.7%, respectively. The specific activity values are in good agreement with the values of this characteristic for enzyme preparations isolated from sunflower (4.6 U/mg) and cucumber (5.8 U/mg) [11]. The maximum elution of the first and second isoforms was observed with the concentration of KCl solution of 60 and 94 mM, respectively (Fig. 2). The homogenous ICL

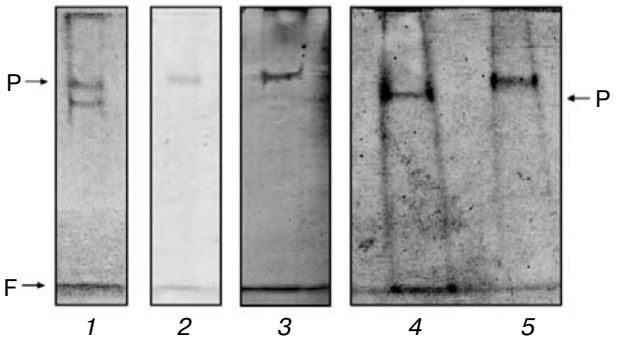


Fig. 1. Electrophoregrams of ICL: *1*, *2*) specific development of ICL from corn scutella and green leaves, respectively; *3-5*) development with silver nitrate of ICL preparations with $R_f = 0.25$ from corn green leaves, ICL_1 with $R_f = 0.29$ from scutella, and ICL_2 with $R_f = 0.25$ from scutella, respectively. Here and in Fig. 3, P is protein band and F is dye front.

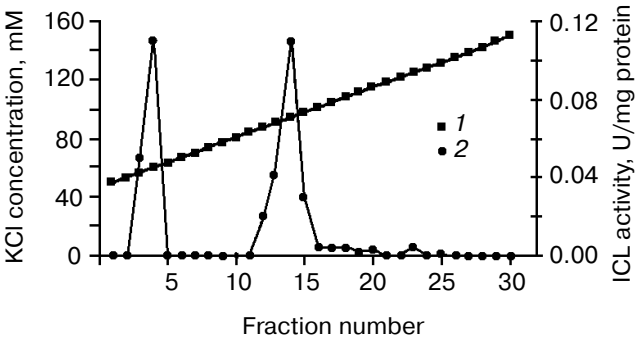


Fig. 2. Elution profile of ICL activity on DEAE-cellulose during desorption by a linear KCl gradient: *1*) ICL activity; *2*) KCl concentration.

Purification of ICL from corn scutella

Purification stage	Volume, ml	Total protein, mg	Specific activity, U/mg	Yield, %
Homogenate	6	51	0.04	100
Fractionation with ammonium sulfate	2	9.6	0.18	91
Gel filtration on G-25	2	9.2	0.19	87
Ion-exchange chromatography on DEAE-cellulose	2	0.023	4.9	5.7
	2	0.025	4.4	5.7

preparation obtained from green leaves of 14-day-old corn seedlings had specific activity of 3.05 U/mg, yield of 10%, and purification degree 113. The enzyme was desorbed from DEAE-cellulose as one peak at potassium chloride concentration of 94 mM.

The electrophoretic analysis of purified preparations and universal staining for proteins and specific development for activity revealed a single protein band (Fig. 1). Thus, corn scutellum was shown to possess two enzyme forms of different electrophoretic mobility: ICL_1 with $R_f = 0.29$ and ICL_2 with $R_f = 0.25$. Green leaves possess only one enzyme form with $R_f = 0.25$ (Fig. 1). Consequently, the R_f value of ICL obtained from the leaves coincides with the value of electrophoretic mobility of the second ICL from corn scutella.

Physicochemical, kinetic, and regulatory characteristics were compared in homogenous ICL preparations obtained from the green leaves and scutella of corn. Gel chromatography on Sephadex G-200 and electrophoresis under denaturing conditions (SDS-PAGE) showed that the ICL from green leaves was similar to ICL_2 and differed in molecular weight of the native isoform and subunits from ICL_1. The isoforms in the corn scutella (ICL_1 and ICL_2) and leaves (ICL) are tetramers with molecular weight of 164-208 kDa. It is interesting to note that the quaternary structure of the ICL from green leaves almost completely coincides in molecular weight and subunit structure with the second enzyme isoform (ICL_2) from corn scutella.

Other molecular structures of ICL have been reported as well: trimeric for the enzyme from germinating pollen of pine *Pinus densiflora* [20], dimeric and pentameric for the enzyme from cucumber [21]. However, the analysis of the literature shows that ICL in most cases is a tetramer protein with molecular weight of subunits ~60 kDa, which differs from the values of 43-47 kDa that we have obtained (Fig. 3) [22]. However, the molecular weight of enzyme subunits may vary substantially. The weight of subunits of ICL isoenzymes calculated by the nucleotide sequence in *A. thaliana* is 36.6 kDa for ICL_1 and 64.1 kDa for ICL_2, with both forms being of homotetramer structure. The similarity of primary structures of the isoenzymes was no more than 21%. Higher similarity (38%) was described for ICL isoenzymes in *Saccharomyces cerevisiae* encoded by the genes located in different chromosomes (5 and 16) (GenBank, Swiss-Prot (http://www.ncbi.nlm.nih.gov)). The corn genome has not been decoded completely; so far, it is known about the presence of one gene encoding ICL of ~50 kDa (http://www.maizegdb.org), which probably corresponds to the ICL_2 values that we have obtained. The maximal weight of oligomeric protein (480 kDa) was obtained for the nematode *Turbatrix aceti*. The molecular weight of the enzyme of higher plants is 264 in flax, 175 in lupine, 260 in cucumber, 140 in castor bean plant, 115 in sunflower, and 270 kDa in pine [1, 10].

The isolated ICL forms are different in the pH optimum for activity, which is 7.5 for ICL_1 and 6.0 for ICL_2 and the ICL of green leaves (Fig. 4). It is reported in the lit-

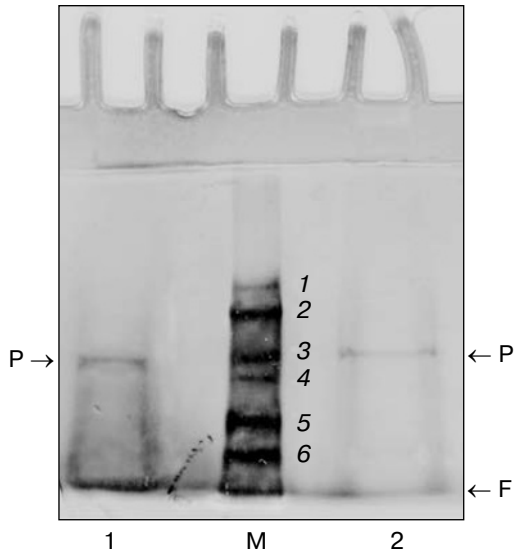
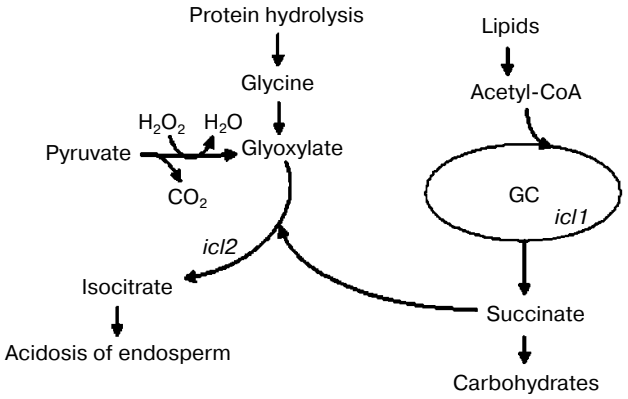


Fig. 3. Determination of molecular weight of corn scutellum ICL subunits by SDS-PAGE: 1) ICL_1; 2) ICL_2. M, marker proteins: *1*) cellulase (94.6 kDa); *2*) BSA (66.2 kDa); *3*) egg albumin (45 kDa); *4*) carboanhydrase (31 kDa); *5*) trypsin inhibitor (21.5 kDa); *6*) lysozyme (14.4 kDa).

erature that in ICL isolated from various sources the value of pH_{opt} varies from 7.0 to 7.5 and depends on the buffer used [23]. However, the most studies were performed with glyoxysomal ICL. It may be supposed that ICL_1 isolated in our work from scutella is a glyoxysomal form that provides GC functioning, while isoforms ICL_2 and ICL are activated during acidification of the cytoplasmic fraction. The glyoxysomal form of isocitrate lyase mainly provides the synthesis of succinate, which is utilized in the Krebs cycle [1]. Activation of mitochondrial oxidative processes seems to play the key role in transformation of reserve lipids into carbohydrates, when it is necessary to transform the succinate formed in the GC into malate for subsequent biosynthesis and homeostasis maintenance of carbohydrates.



Scheme of participation of ICL isoforms in regulation of cell metabolism in corn scutella

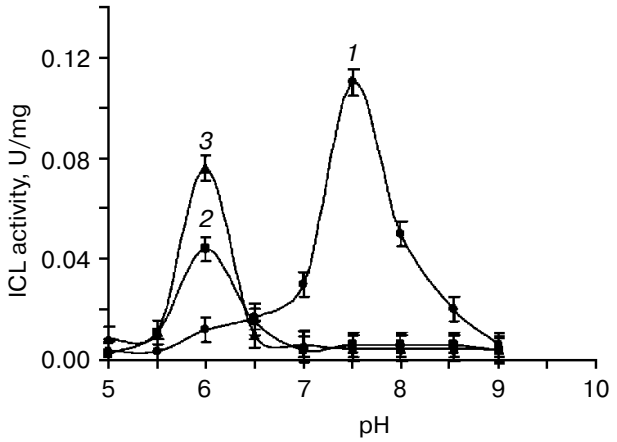


Fig. 4. Dependence of the activity of ICL isoforms on pH: *1*) ICL_1; *2*) ICL_2; *3*) ICL from green leaves.

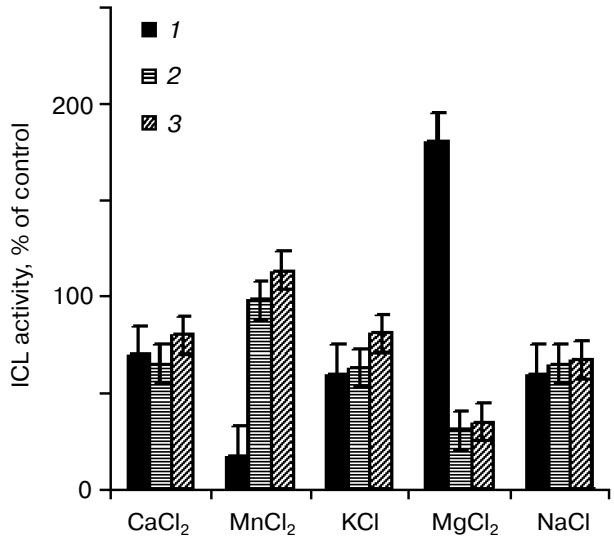


Fig. 5. Effect of metal ions (5 mM) on the activity of ICL isoforms from corn scutella and green leaves: *1*) ICL_1; *2*) ICL_2; *3*) ICL from green leaves.

Certain differences are also revealed on analysis of K_m values of different isoforms of isocitrate lyase. ICL_1 from corn scutella is characterized by the highest K_m value for isocitrate (56 μM), while ICL_2 and ICL have values of 83 and 100 μM, respectively.

It is interesting that the glyoxysomal form of isocitrate lyase (ICL_1) is an Mg-dependent enzyme, while ICL_2 and ICL are activated by Mn^{2+}. Thus, these enzyme forms are Mn-dependent. Other cations (Ca^{2+}, K^+, and Na^+) have little effect in the regulation of activity of ICL isoforms (Fig. 5).

ICL from green leaves by most characteristics (R_f, quaternary structure, pH_{opt}, affinity to substrate, activation by ions, etc.) is very similar to ICL_2 from corn scutella. This suggests that these forms perform functions not associated with GC. The previously described extraglyoxysomal ICL from green leaves is involved in metabolizing photorespirative glyoxylate. We suppose that the second ICL isoform in corn scutella (ICL_2) also metabolizes organic acids, in particular, glyoxylate, using the synthase reaction (see Scheme) [10]. Glyoxylate utilization causes acidification (acidosis) of endosperm, which is needed for effective hydrolysis of reserve substances [24].

In higher plants, reports on ICL functioning beyond the GC most often concern studies of aging leaves, where this enzyme may participate in mobilization of degrading lipids [7]. Such, for example, are the data of Hunt et al. [25] on the involvement of ICL in the biosynthesis of glycine and serine in pea leaves. Millerd et al. [26] have found ICL in oxalis seedlings in the absence of malate synthase and shown the possibility of participation of ICL in biogenesis of oxalic acid. It is supposed that ICL may also play some role in oxalate accumulation in the leaves of other plants. Besides, it has been shown that in tobacco leaves the extraglyoxysomal ICL (associated with the mitochondrial fraction) provides the formation of glycolate from pyruvic acid [27].

The analysis of the cited works leads to a conclusion that the role of ICL in living organisms (in particular, higher plants) is very diverse and certainly not confined to participation of the enzyme in the GC. It seems that the enzyme functioning in other metabolic pathways is not a side effect of glyoxysomal ICL but there is another form of the enzyme different in properties and localization from the glyoxysomal one.

Thus, supposedly extraglyoxysomal and glyoxysomal ICL forms, vastly different in properties, have been found in plant cells. The presence of at least two ICL-encoding genes in the genome of *Arabidopsis* and other organisms suggests that the revealed enzyme forms are isoenzymes, i.e. genetically differently determined proteins, the expression of which is regulated independently.

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