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Isocitrate Lyase from Germinating Soybean Cotyledons: Purification and Characterization

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ABSTRACT

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Isocitrate lyase (E.C. 4.1.3.1) was purified from the cotyledons of 7-d-old soybean seedlings. Three molecular forms were detected with *pI* values of 6·46, 6·25 and 6·0. The main form (*pI* = 6·46) had an approximate Mr of 130 000, a pH optimum of 8·0, a K_m (isocitrate) close to 2·0 mol m^{-3} and a molecular activity of 615 min^{-1} at 25 °C. The purified enzyme is not a glycoprotein and is heat labile.

Key words—Isocitrate lyase, soybean.

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INTRODUCTION

In germinating oleaginous seeds, net gluconeogenesis from reserve lipids involves the glyoxylate cycle. The enzyme isocitrate lyase (*threo*-Ds-isocitrate glyoxylate-lyase, E.C. 4.1.3.1) is specific for this metabolic pathway, and catalyses the cleavage of isocitrate into succinate and glyoxylate. The glyoxylate cycle is also present in various micro-organisms, which can thus use 2-carbon compounds for the net synthesis of glucose. Isocitrate lyases from various sources have already been described, but to date the enzyme from soybean cotyledons has not been extensively studied. This report describes its main physico-chemical and reactional characteristics.

MATERIALS AND METHODS

Chemicals

Threo-Ds(+)-isocitric acid and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma. Gel filtration, ion exchange media (Sepharose CL-6B, DEAE- and CM-Sepharose CL-6B, Sephacryl S-300 Superfine) and the electrophoresis calibration kit were products of Pharmacia. Materials for electrofocusing (Ultrodex and Ampholines) were obtained from LKB. Mr values were determined using the following proteins as standards: equine myoglobin (17 800), human albumin (69 000), human γ-globulin (150 000) and porcine thyroglobulin (660 000) (all from Serva); soybean lipoxidase (108 000) from Sigma and bovine glutamate dehydrogenase (350 000) from Merck. All other chemicals were obtained in the purest forms available from commercial sources.

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Abbreviations: BSA: bovine serum albumin; DTT: dithiothreitol; PMSF: phenylmethylsulphonyl fluoride; SDS: sodium dodecyl sulphate.

Plant material, germination conditions and flour preparation

Soybeans (*Glycine max.* L., cv. Maple arrow) were provided by Schweizer Samen AG, CH-3600 Thun, Switzerland. The seeds were germinated on moist cotton wool for 7 d in the dark without prior imbibition and surface sterilization. The temperature was initially 30 °C, but was lowered to 20 °C after 6 h. No fungal contamination developed, and the cotyledons were freeze-dried after removal of the teguments, epicotyls and radicles. The dried cotyledons were ground to a powder (soyflour) in a Cyclotec mill (1-mm grid) and defatted in a Sohxlet apparatus (4 h) using petroleum ether (boiling range 40 °C-60 °C).

Protein extraction

Soyflour was extracted with eight times its weight of distilled H_2O (Wolf and Briggs, 1956) containing 5·0 mol m^{-3} DTT and 1·0 mol m^{-3} of the serine-protease inhibitor PMSF (Pinzauti, Giachetti, and Vanni, 1983; Jameel, El-Gul, and McFadden, 1984). The mixture was homogenized with a Polytron (Kinematica, GmbH) for 1 min followed by 1·5 h moderate stirring at room temperature. Insoluble particles were removed by centrifugation for 30 min at 27 000 × *g* and 4 °C. Clear extracts were obtained after 15 h dialysis at 4 °C against 50 mol m^{-3} Tris-HCl buffer (pH 7·2) containing 5·0 mol m^{-3} DTT, 10 mol m^{-3} $MgCl_2$ and 1·0 mol m^{-3} PMSF.

Enzyme assay

Isocitrate lyase was assayed at pH 8·0 and 25 °C, as described by Dixon and Kornberg (1959) but using 5·0 mol m^{-3} DTT instead of 2-mercaptoethanol, 3·3 mol m^{-3} phenylhydrazine-HCl, 10 mol m^{-3} $MgCl_2$ and 5·0 mol m^{-3} *threo*-Ds(+)-isocitric acid (Tester, 1976). One unit of enzymatic activity (*U*) was the amount that formed one nanomole of glyoxylic phenylhydrazone per min. The molar extinction coefficient used was $16·8 \times 10^3$ M^{-1} cm^{-1} at 324 nm (Turian and Kobr, 1965).

Enzyme purification

The 4-step procedure involved classical methods (see Results and Discussion).

pI determination

Electrofocusing on Ultrodex gels was carried out for 14 h following the manufacturer's instructions, between pH 4·9 and 7·5, and initially at 15·5 mA and 500 V.

Thermal inactivation of the enzyme

Thermal inactivation of isocitrate lyase was achieved by incubating the enzyme for 5 min at various temperatures in 50 mol m^{-3} Tris-HCl buffer (pH 7·2). The remaining activity was then assayed under standard conditions.

Other analytical methods

Protein contents were determined by the method of Bradford (1976), using BSA as the standard. Mr values were determined using a calibrated Sephacryl S-300 Superfine column (0·9 cm × 53 cm), equilibrated with 100 mol m^{-3} Tris-HCl buffer (pH 7·2). The method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) was used as a test for glycoprotein. Electrophoresis in 12·7% polyacrylamide gels containing 0·1% SDS was carried out after denaturation of the enzyme in presence of 5% mercaptoethanol. The electrode buffer was 50 mol m^{-3} Tris-glycine (pH 8·3). Gels were calibrated with standard proteins. The method was essentially as described by Weber and Osborn (1969).

RESULTS AND DISCUSSION

Enzyme purification

Solid $(NH_4)_2SO_4$ was added to the dialysed aqueous crude extract to a concentration of 25% saturation. After 1 h moderate stirring at 4 °C, the precipitate was removed by centrifugation (25 min at 27 000 × *g*). The supernatant liquid was made to 50% saturation with $(NH_4)_2SO_4$ and the precipitate recovered by centrifugation was resuspended in a minimum volume of 50 mol m^{-3} Tris-HCl buffer pH 7·2. Proteins in this solution were fractionated by gel filtration on Sepharose CL-6B (50 mol m^{-3} Tris-HCl pH 7·2). A single symmetrical peak of enzymatic activity was eluted in fractions indicating a protein of Mr in the range 100 000–140 000. The

active fractions were concentrated using Amicon Diaflo cells (YM-10 membranes; the same technique was used to concentrate the fractions obtained from the ion-exchange columns).

Anion exchange chromatography separated the isocitrate lyase activity into two distinct molecular species (peaks Ia and IIa on Fig. 1). Most of the enzymatic activity was eluted from the column in the buffer alone as an asymmetrical profile (see also Table 1 below), but further activity was recovered between 130 and 200 mol m^{-3} NaCl during subsequent gradient elution.

The pooled fractions from peak Ia (Fig. 1) were dialysed overnight against 25 mol m^{-3} sodium-phosphate buffer (pH 6·2). Insoluble material was removed by centrifugation

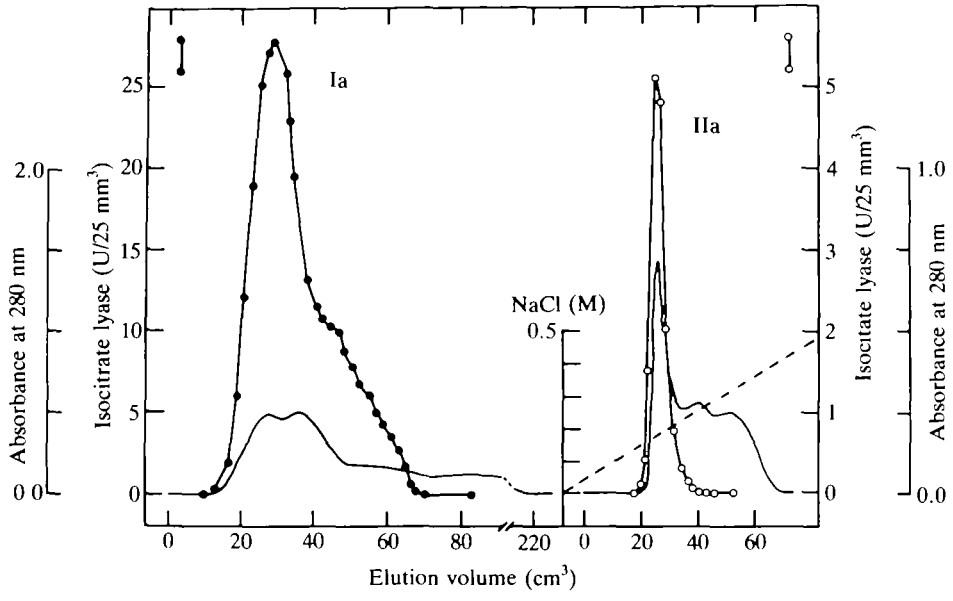

FIG. 1. DEAE-Sepharose CL-6B chromatography in 50 mol m^{-3} Tris-HCl buffer (pH 7·2). Column dimensions. 1·6 cm × 15 cm. The sample volume was 6·0 cm^3; the flow rate was 0·4 cm^3 min^{-1} and 2·0 cm^3 fractions were collected.

TABLE 1. *Purification of isocitrate lyase from soybean cotyledons*

Step	Total protein (mg)	Activity		Purification (-fold)	Recovery (%)
		Total (*U*)	Specific (*U*/mg)		
Crude extract	1550	42 000	27	—	100
Dialysed extract	935	40 000	43	1·6	95
Precipitation (25-50% ammonium sulphate saturation)	423	26 400	62	2·3	63
Sepharose CL-6B	168	21 500	128	4·7	51
DEAE-Sepharose					
(peak Ia)	5·6	14 000	2 500	92·6	33
(peak IIa)	0·64	700	1 094	40·5	1·7
CM-Sepharose CL-6B					
(peak Ib)	2·8	9 070	3 240	120	22

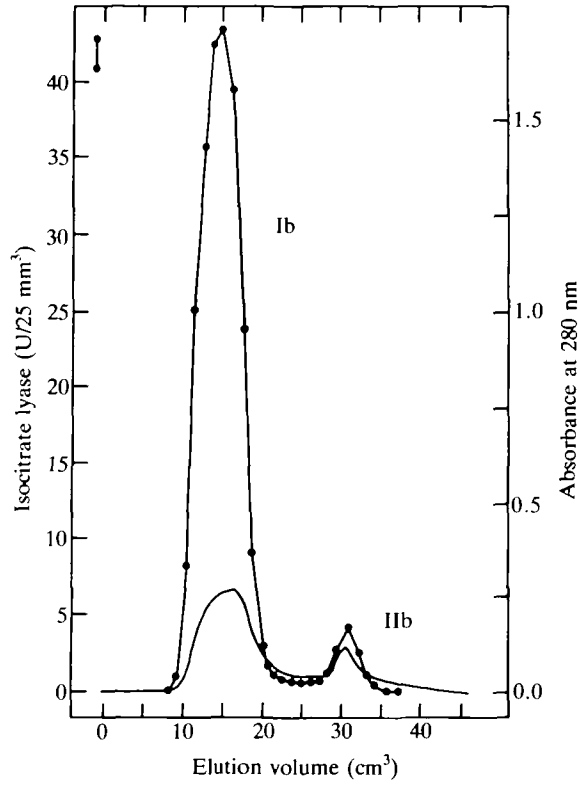

FIG. 2. CM-Sepharose CL-6B chromatography in 25 mol m^{-3} sodium-phosphate buffer (pH 6·2). Column dimensions: 0·9 cm × 30 cm. The sample volume was 6·0 cm^3; the flow rate was 0·12 cm^3 min^{-1} and 1·0 cm^3 fractions were collected.

(27 000 × *g*, 4 °C) and the supernatant liquid was applied to a cation exchange column. Active proteins were eluted (peaks Ib and IIb, Fig. 2) with phosphate buffer alone and no additional activity was eluted on application of the NaCl gradient.

Table 1 summarizes the purification obtained by the various steps. Table 2 compares recent purifications achieved by various authors for isocitrate lyases extracted from higher plant cotyledons. Unless otherwise indicated, the properties of the purified enzyme were investigated using the pooled fractions obtained from peak Ib seen on Fig. 2.

Physico-chemical properties

Two peaks of isocitrate lyase activity with *pI* values of 6·46 and 6·25 were obtained by electrofocusing protein from peak Ia, Fig. 1. By contrast, electrofocusing of protein from peak IIa showed the presence of active protein with a *pI* of 6·0. The electrofocusing procedure resulted in a dramatic loss of enzyme activity and could not, therefore, be routinely used as a purification step.

The purified enzyme was not a glycoprotein. This may explain its significant heat sensitivity. When incubated for 5 min at pH 7·2 the enzyme was rapidly inactivated when temperature exceeded 40 °C.

Gel filtration of the purified enzyme on a calibrated Sephacryl S-300 Superfine column indicated a Mr of 130 000. This is close to the values obtained for isocitrate lyases extracted from seeds of castor bean (140 000, Malhotra and Srivastava, 1982) and lupin (145 000,

TABLE 2. *Comparison of recoveries and purification factors obtained by various authors for the purification of higher plant isocitrate lyases*

Material	Recovery (%)	Purification (-fold)
Lupin (Vincenzini *et al.*, 1980)	1·4	87
Cucumber (Frevert and Kindl, 1978)	8·5	106
Flax (Khan *et al.*, 1977)	14	111
Pine (Pinzauti *et al.*, 1986)	16	126
Watermelon (Jameel *et al.*, 1984)	36	97
Soybean (present study)	22	120

Vincenzini, Nerozzi, Vincieri, and Vanni, 1980). By contrast, higher values have been reported for the enzyme from cucumber cotyledons (255 000, Frevert and Kindl, 1978), flax seedlings (264 000, Khan, Saleemuddin, Siddiqi, and McFadden, 1977) or watermelon cotyledons (277 000, Jameel *et al.*, 1984). Similarly, the enzyme from various micro-organisms also appears to have larger Mr values e.g. *Chlorella pyrenoidosa* (170 000, John and Syrett, 1967) and *Pseudomonas indigofera* (222 000, Rao and McFadden, 1965).

The enzymes from watermelon, cucumber and flax cotyledons are reported to be tetrameric with subunit Mr's ranging from 64 000 to 67 000 (Jameel *et al.*, 1984; Frevert and Kindl, 1978; Khan *et al.*, 1977). Our own preliminary results (SDS electrophoresis) suggest that the enzyme extracted from soybean cotyledons could be dimeric with an approximate subunit Mr of 70 000. Thus, isocitrate lyases of higher plants may be made up of an even number of 64–70 kDa subunits.

Reactional characteristics

The pH activity profile was investigated in the range 7·0–9·0, using 50 mol m^{-3} sodium-phosphate and Tris-HCl buffers. The optimum pH was 8·0, which is similar to the values reported for isocitrate lyases from other sources (pH 7·5–7·6, John and Syrett, 1967; Khan *et al.*, 1977; Rao and McFadden, 1965). The values measured at pH 7·5 and 8·5 were approximately 75% of the value at the optimum.

K_m determination was carried out at pH 8·0, using sodium-phosphate and Tris-HCl buffers respectively, according to the methods of Lineweaver and Burke (1934), Hanes (1932) and Eisenthal and Cornish-Bowden (1974). The average K_m values for isocitrate were 1·8 mol m^{-3} in phosphate buffer and 2·1 mol m^{-3} in Tris-HCl buffer. This indicates a lower affinity toward isocitrate, compared to the values obtained for isocitrate lyases extracted from *Pseudomonas indigofera* (0·82 mol m^{-3}, McFadden and Howes, 1963), flax seedlings (0·29 mol m^{-3}, Khan *et al.*, 1977) and *Chlorella pyrenoidosa* (23 mmol m^{-3}, John and Syrett, 1967). The molecular activity (k_{cat}) at 25 °C was 615 min^{-1}, as calculated from the V_{max} value and the estimated Mr of 130 000.

The characteristics of the inhibition of isocitrate lyase from soybean cotyledons by several gluconeogenesis metabolites were recently reported in a separate paper (Ruchti and Widmer, 1986).

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