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THE IDENTIFICATION AND PROPERTIES OF A NATURALLY OCCURRING INHIBITOR OF MALIC ENZYME

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Key Word Index—*Solanum tuberosum*; Solanaceae; malic enzyme inhibitor; oxalic acid.

Abstract—The inhibitor of malic enzyme present in potato tubers has been identified as oxalic acid. Oxalic acid proves to be a particularly potent inhibitor with a $K_I = 50$ μM. A kinetic analysis indicates that inhibition is not due to chelation of Mg^{2+} and suggests that oxalate binds tightly to malic enzyme after NADPH has been bound.

INTRODUCTION

Malic enzyme (L-malate-NADP oxidoreductase (decarboxylating) EC 1.1.1.40) has been isolated from a number of plants but has been reported absent from potato tubers [1] and the roots of flood tolerant plants [2]. Subsequent work has demonstrated the presence of malic enzyme in such plants [3] and results obtained with potato extracts demonstrated the presence of an inhibitor of the enzyme [4]. In this paper we report the identification of this inhibitor as oxalic acid and report on the kinetics of inhibition.

RESULTS

Partial purification of the inhibitor

A number of methods were initially surveyed such as liquid–liquid ether extraction, lead precipitation and silica gel chromatography. However ion exchange chromatography on Dowex-2 proved the most reliable and rapid method for the partial purification of the inhibitor (Table 1). Details of the procedure are given in the experimental. Since the procedure would be expected to concentrate organic acids, malate and citrate were also assayed in the eluates from the Dowex-2 columns.

Identification of the inhibitor

The peak fractions of the inhibitor emerging from the Dowex-2 column were concentrated and on standing overnight at 0° crystals were obtained which were shown to be oxalic acid (mp, IR and UV) reaction with diphenylamine [7] and by PC (R_f 0·4) using *t*-amylalcohol–HOAC–H_2O (4:1:5) as the solvent system and ferric ammonium sulphate (0·5%) in EtOH (70%) and aq ammonia (10%) for colour development [8].)

Table 1. Purification of the inhibitor of malic enzyme from potato tubers

Fraction	Volume (ml)	Units of inhibitor ($\times 10^{-3}$)	Recovery (%)
Crude extract	982	57·8	100
After Dowex 50	1220	48·0	82·9
Dowex 2 (first elution)	72	35·5	61·4
Dowex 2 (second elution)	60	24·1	41·5

Several estimates of the molecular weight of the inhibitor were made by molecular sieving, values varying between 300–600 being obtained by the method of Andrews [6] using different gels. When subsequent work indicated that the inhibitor was oxalic acid, we rechecked the determinations using ^{14}C-oxalic acid as a marker and again obtained a high anomalous molecular weight with the inhibitor and ^{14}C oxalic acid emerging in the same fractions.

The identification was confirmed by using a different isolation method [9] and by the IR spectrum of the calcium salt.

Evidence that oxalic acid is the only heat stable inhibitor of malic enzyme present in potato tubers

It could be argued that potato tubers contain more than one heat stable inhibitor of malic enzyme and that the purification procedure concentrated only one of them. Alternatively the purified inhibitor fractions contain another innhibitor besides oxalic acid. To eliminate these possibilities we have measured the amount of oxalic acid in extracts of potato tubers by isotope dilution, then compared the degree of inhibition of malic enzyme produced by a given volume of extract with the inhibition produced by the amount of oxalic acid present in the same volume. The results with the purified inhibitor were consistent with oxalate being the only inhibitor present. The results with the crude extract were consistent with oxalate being the only inhibitor present but were complicated by the presence of other organic acids which activate malic enzyme [4].

Kinetic of inhibition of malic enzyme by oxalic acid

Malic enzyme from potato tubers has been shown to exhibit allosteric properties [4]. To avoid difficulties of interpretation we have studied the kinetics of inhibition at pH 7·0, since at this pH the kinetic of the enzyme are approximately Michaelis–Menten. When malic

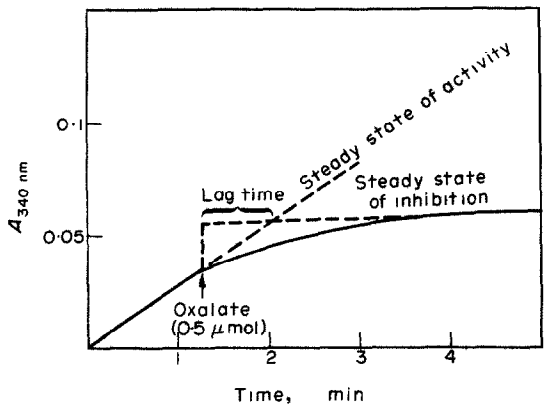

Fig. 1. Plot of rate of NADPH formation by malic enzyme before and after the addition of oxalate. The malic enzyme inhibitor was assayed by measuring the reduction in rate of reaction on addition of the inhibitor at the time indicated to an assay system (3 ml) containing L-malate (3·3 mM), $MgCl_2$ (1·7 mM) Mops buffer (pH 7·0 100 mM), NADP (0·17 mM) and malic enzyme purified from potato tubers [5]. (A unit of enzyme activity is defined as the amount of enzyme producing 1 μmol product/min; equivalent to E_{340nm} of 2·03/min in a 1 cm cell). A unit of inhibitor is defined as the amount which produces 50% inhibition in the standard assay of malic enzyme.

enzyme is assayed under standard conditions and oxalate added, the inhibitory response shows two phases—an initial phase of relatively slight inhibition followed by a phase of greater inhibition (Fig. 1). When the enzyme was assayed in the direction of pyruvate reduction, the inhibition by oxalate was immediate. This observation, together with other kinetic data suggest that before oxalate can exert its inhibitory effects, the enzyme must bind NADPH. If this proposition is correct, it follows: (a) that if oxalate is added at various time intervals after the start of the reaction inhibition should increase, as the time before the addition of oxalate is increased; (b) that the inhibition produced by oxalate at the start of the reaction should be increased by adding increasing amounts of NADPH at the start of the reaction. Both predictions were confirmed (Fig. 2).

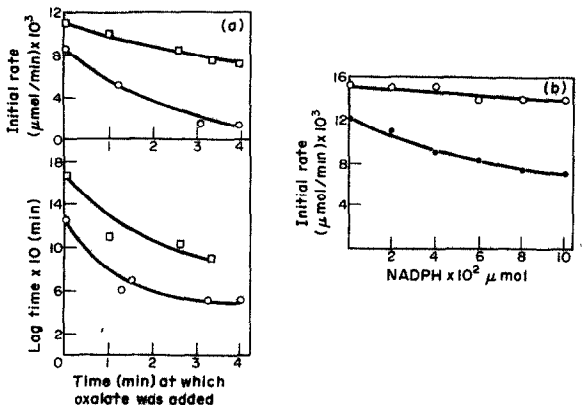

Fig. 2. Effect of product formation on the inhibition of malic enzyme by oxalic acid. (a) Effect of time at which oxalate was added. (lag time is measured as shown in Fig (1). □——□ oxalate 33 μM, ○——○ oxalate 165 μM. (b) Effect of adding product (NADPH) at time t_0 ●——● oxalate 33 μM, ○——○ no oxalate.

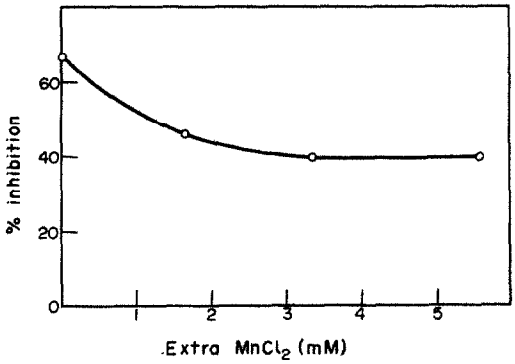

Fig. 3. Effect of additional Mn^{2+} on the inhibition of malic enzyme by oxalate (0·033 mM). The enzyme was assayed under standard conditions and extra $MnCl_2$ added as indicated.

The effect of oxalate on the activity of malic enzyme has also been examined with respect to varying concentrations of substrates. The results show that in the direction of pyruvate carboxylation, oxalate shows non-competitive inhibition with respect to pyruvate; the inhibition with respect to NADPH is uncompetitive at low concentration. In the direction of malate decarboxylation, oxalate shows non-competitive inhibition with respect to malate but competitive inhibition with respect to NADP.

Since oxalic acid is a strong chelating agent it is possible that the inhibitory properties of oxalate are due to removing Mn^{2+}. That this proposition is not true, can be seen from Fig. 3 which shows the oxalate inhibition persists in the presence of a gross excess of Mn^{2+}. Similarly, the kinetics of competitive inhibition with respect to NADP are not significantly affected by the addition of an extra amount of Mn^{2+} equivalent to the oxalate added. However, it should be noted that these experiments do not exclude a chelation effect in which Mn remains bound to the enzyme.

Oxalic acid in flood tolerant plants

The reported absence of malic enzyme in a number of tissues could be due to the presence of oxalic acid in the crude enzyme extracts. In view of the anomalous behaviour of oxalic acid in molecular sieving, it is possible that plant extracts prepared for assay by desalting with Sephadex could be contaminated with oxalic acid. However, careful desalting of preparations on Sephadex-G25 columns effectively separate malic enzyme and oxalic acid.

Discussion

The results reported in this paper establish that the inhibitor of malic enzyme present in potato tubers is oxalic acid. It is possible that reports indicating the absence of malic enzyme from some other plants are due to the presence of this acid in the crude extracts used for enzyme assay. The complete separation of oxalic acid and malic enzyme on a column of Sephadex G-25 suggests that this or a similar method should be a standard procedure in the assay of the enzyme from plants.

Oxalic acid is widely distributed in plants and commercial preparations of L-malic acid are frequently contaminated with oxalate [10]. Ranson [11] suggests that it is probable that oxalic acid accumulates "at a place,

or in a form which renders it immune from further metabolism". Thus, if it is located in the vacuole and malic enzyme in the cytoplasm, inhibition does not take place *in vivo*. On the other hand, oxalic acid is probably synthesized in the cytoplasm and since malic enzyme is inhibited by very low concentrations, the possibility of an *in vivo* effect on malic enzyme cannot be excluded.

The kinetics of the potato malic enzyme have been shown to be (in the nomenclature of Cleland [12]) ordered Bi Ter, with NADP binding before malate and NADPH being last to leave the enzyme [5]. The kinetics of inhibition by oxalate are consistent with a mechanism whereby oxalate combined with the enzyme to give a dead-end complex only after NADPH has been bound. Such a mechanism predicts the observed pattern of inhibition, oxalate showing non-competitive inhibition with malate. The analysis does not establish the mechanism whereby oxalate combines with the enzyme. However, the formation of an E–NADPH–Mn^{2+}-oxalate complex seems a likely possibility and evidence for this association could be obtained by equilibrium dialysis.

The anomalous behaviour of oxalic acid during chromatography on Sephadex G-10 and G-15 misled us in our early attempts to identify the inhibitor. Kun [13] was also misled by an anomalous molecular weight for his cytoplasmic metabolic factor (CMF) obtained by chromatography on Sephadex G-25. The adsorption of certain molecules on Sephadex gels is a likely possibility and to that extent molecular sieving is likely to produce an underestimate of molecular weight. The mechanism of excluding certain small molecules from Sephadex gels is less obvious and the overestimation of molecular weights was not expected. However the anomalous behaviour of oxalic acid and CMF on Sephadex gels suggests caution in the interpretation of elution patterns of low M.W. acids.

EXPERIMENTAL

Chemicals. NADP and NADPH were obtained from Sigma Chemical Co., London, Sephadex gels from Pharmacia Uppsala, oxalic acid-^{14}C from the Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of the best grades obtainable commercially.

Malic enzyme. The potato enzyme purified according to Nascimento and Davies [5] was stable for several months when stored at −15°.

Purification of the inhibitor from potato tubers. Potato tubers (2 kg) were peeled, cut into segments and homogenized with 500 ml of 1 mM mercaptoethanol for 30 sec in a Kenwood homogeniser. The extract was squeezed through 2 layers of muslin to remove starch and cell debris then boiled for 15 min. The extract was cooled to 5° then centrifuged at 13000 *g* for 20 mins. The clear supernatant (980 ml) was passed through a column (8·5 × 20 cm) of Dowex 50 (H^+ form) to remove bases and amino acids. The eluate and washings (1220 ml) were then applied to a column (3 × 40 cm) packed with Dowex 2 (formate form). The column was eluted by applying a linear concentration gradient obtained by placing 250 ml H_2O in the mixing cylinder and an equal volume of sodium formate (3M) in the reservoir. Fractions (6 ml) were collected for assay, the peak fractions containing the inhibitor were combined (72 ml) and passed through a column (8·5 × 20 cm) of Dowex 50 (H^+) to remove Na^+. Formic acid was removed on a rotary evaporator and the resulting solution adjusted to pH6 with NaOH (1M) before being transferred to a Dowex 2 (formate form) column (3 × 40 cm). The inhibitor was eluted as described before, the peak fractions compound, passed through a column (8·5 × 20 cm) of Dowex 50 (H^+) and concentrated on a rotary evaporator. The concentrated solution was stored overnight at 0° to yield white crystals.

Purification of the inhibitor on Dowex-2 (acetate using Mg acetate as elutant [9]. The potato extract (500 ml) was passed through Dowex 50 (H^+) as described above and the eluate adjusted to pH 6·0 with NaOH before being passed through a column (1·2 × 100 cm) packed with Dowex-2 (acetate). The inhibitor was eluted with 2·5 l. Mg acetate (0·2M pH 3·9) and the fractions (6 ml) assayed for inhibitor, malate and citrate. The peak fractions were pooled, passed through a Dowex 50 (H^+ form) column (3 × 20 cm) and 20 ml of acetic acid (6M) added to the eluate. $CaCl_2$ (20% W/V) was slowly added until a white precipitate was obtained. After standing for 12 hr, the ppt. was filtered, washed with H_2O and dried at 100° for 6 hrs.

Substrate assays. Malic acid and citric acid were assayed as described by Williamson and Corkey 14.

Measurement of ^{14}C-oxalic acid. ^{14}C-oxalic acid were counted in a Packard liquid Scintillation Spectrometer using toluen–Triton–PPO 1000/500/7 v/v/w as the scintillant.

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