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Acetyl-CoA carboxylase: a rapid novel assay procedure used in conjunction with the preparation of enzyme from maize leaves

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Acetyl-CoA carboxylase is known to be the site of action of two groups of grass-selective herbicides: the aryloxyphenoxypropionates and the cyclohexanediones. This enzyme was extracted from maize leaves, and a novel, highly sensitive assay system developed, utilising measurement of phosphate to estimate enzyme activity. This assay system was verified by using inhibition by fluazifop and avidin to compare it with the more widely used ^{14}C assay. K_m values for acetyl-CoA were measured, and inhibition kinetics investigated.

Acetyl-CoA carboxylase; Fluazifop; Aryloxyphenoxypropionate; Cyclohexanedione; (Maize leaves)

1. INTRODUCTION

Acetyl-CoA carboxylase (acetyl-CoA carbon dioxide ligase (ADP-forming), EC 6.4.1.2) is the first step committed to the pathway of fatty acid biosynthesis, converting acetyl-CoA to malonyl-CoA. The enzyme from plants has become the focus of attention recently as the site of action of two separate chemical groups of grass-selective herbicides. Thus, the aryloxyphenoxypropionic acids [1–5] are inhibitory to the monocot leaf enzyme with considerably less activity against the dicot enzyme. It is known that only the R-enantiomer inhibits the enzyme [4,5], and kinetic analyses have shown non-competitive inhibition with respect to all three substrates, acetyl-CoA, MgATP and bicarbonate by one of the herbicides (diclofop) in this series [4].

Likewise, the cyclohexanedione herbicides also inhibit acetyl-CoA carboxylase [1,2,6,7] with a greater inhibition of the enzyme from monocots than dicots. Thus, the primary basis for selectivity by both chemical types appears to be the extent to which acetyl-CoA carboxylase is inhibited in the two groups of plants.

The enzymic reaction is a two-step process. The first involves the ATP-dependent carboxylation of biotin in a biotin carboxyl carrier protein (BCCP) by biotin carboxylase, which results in the liberation of ADP and phosphate. In the second step the carboxyl group is transferred to acetyl-CoA by transcarboxylase activity to produce malonyl-CoA:

$$ATP + CO_2 + BCCP \longrightarrow CO_2\text{-}BCCP + ADP + P_i$$

$$CO_2\text{-}BCCP + \text{acetyl-CoA} \longrightarrow \text{malonyl-CoA} + BCCP$$

The relationship of these individual reactions to the structure of the acetyl-CoA carboxylase enzyme varies according to source. In the enzyme from *E. coli* there are 3 different polypeptide chains containing the carboxylase, BCCP, and transcarboxylase, respectively, and these 3 components can be separated from one another [8]. In contrast, the components in the animal enzyme are in a single polypeptide chain which by itself is inactive, but when aggregated into polymers in the presence of citrate produce the active form of the enzyme [9].

The organisation of the enzyme from plants is intermediate between that of bacteria and animals in that the plant forms can be dissociated into high molecular weight subunits of approximately 220000, but the 3 catalytic domains are present on each polypeptide subunit [10]. As a consequence of this, and also the two-site kinetic mechanism proposed by Finlayson and Dennis [11], we have anticipated that the two reaction steps might remain tightly coupled, and so allow us to use the production of phosphate as an easily manageable assay procedure, particularly for rapid kinetic analyses.

In this study the use of a highly sensitive phosphate assay has been compared with the more widely employed [^{14}C]bicarbonate assay to determine acetyl-CoA K_m values, and inhibition kinetics by the herbicide fluazifop.

2. MATERIALS AND METHODS

Acetyl-CoA carboxylase was extracted from freshly harvested 10-day-old maize (*Zea mays* var. Pioneer 3183) leaves which were

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homogenised in a Waring blender in ice-cold buffer A, pH 8.0, containing 0.1 M Tris, 20 mM mercaptoethanol, 1 mM EDTA, 0.5% Triton X-100, 1 mM benzamidine, 10 mM magnesium chloride and 20% glycerol. The extract was centrifuged at 27500 × g for 30 min and the resulting supernatant filtered through 50 μm filter cloth followed by precipitation with ammonium sulphate between 35 and 45%. The final pellet was resuspended in buffer B, pH 8.0, containing 10 mM Tris, 20 mM mercaptoethanol, 1 mM EDTA, 1 mM benzamidine, 10 mM magnesium chloride and 20% glycerol, and passed down a gel filtration column containing Sephacryl S300HR pre-equilibrated with buffer B but containing only 10% glycerol. Active fractions were pooled and loaded onto a preparative Mono-Q ion exchange column (attached to an FPLC system), previously equilibrated with buffer containing 10 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, 1 mM benzamidine, 10 mM magnesium chloride, and 10% glycerol, pH 8.0. Acetyl-CoA carboxylase was eluted with a KCl gradient between 0.2 and 0.5 M. Protein concentrations of enzyme preparations were measured using the method of Bradford [13].

During the isolation procedure the enzyme was assayed by the method of Nikolau et al. [14]. A final assay volume of 200 μl contained 0.1 M Tricine-KOH (pH 8.0), 0.5 mM ATP, 2.5 mM magnesium chloride, 50 mM potassium chloride, 30 mM $NaH^{14}CO_3$ (0.27 μCi), 1 mM dithiothreitol, 0.3 mM acetyl-CoA (omitted from controls), and up to 100 μl enzyme preparation. Samples were pre-incubated at 30°C for 3 min prior to addition of acetyl-CoA to start the reaction, followed by vigorous mixing and incubation at 30°C for 10 min. The reaction was stopped by the addition of 50 μl 6 M HCl, followed by further vigorous mixing. For the ^{14}C assay, a 75 μl aliquot was removed and dried on a 3.2 cm diameter Whatman GF/A filter. The radioactivity was counted in Optiphase scintillation fluid, in a Packard 460 scintillation counter. In view of the difficulties of calibrating dpm with nmoles malonyl-CoA, all results of the radioactive assay are given as dpm incorporated into malonyl-CoA.

In establishing an alternative assay procedure, the remaining incubation mixture was used for phosphate determination, using a modified method based on that of Hess and Derr [15]. A Malachite Green solution was prepared by mixing 3 parts of 0.045% Malachite Green hydrochloride in distilled water with 1 part 4.2% ammonium molybdate in 4 M HCl. The solution was left to stand for 30 min, then filtered through an 'Acrodisc' 0.45 μm PTFE filter. Triton X-100 (1.5%) in distilled water was added to a final concentration of 0.0375%, and 750 μl of this solution was added to the remaining incubation mixture. After allowing this mixture to stand for 3 min, 150 μl 34% citric acid was added, and samples were read at 630 nm after standing for a further 60 min. Experimental samples were compared with standards containing 1–10 nmol inorganic phosphate.

Inhibition experiments were carried out using the active R-enantiomer of fluazifop. This is the active acid component of the grass-specific herbicide fluazifop-butyl (marketed under the trade name 'Fusilade') that has been shown to inhibit acetyl-CoA carboxylase in barley, but not in peas [5]. Results of inhibition experiments are given in terms of IC_{50}. This is defined as the concentration of inhibitor required to give 50% inhibition. IC_{50} values and K_m values were calculated using a FIT programme (Dr D.K. Lawrence, ICI Agrochemicals).

[^{14}C]Sodium carbonate (2–10 mCi·mmol^{-1}), standard phosphorus solution and ATP were obtained from Sigma Chemical Co. Acetyl-CoA was obtained from Boehringer Mannheim. Sephacryl S300 HR and the preparative Mono-Q ion exchange column were obtained from Pharmacia LKB Biotechnology.

Table 1

Purification of maize acetyl-CoA carboxylase

Enzyme fraction	Specific activity (dpm/mg)	% Recovery	Overall purification
(1) Crude extract	1125	–	–
(2) $(NH_4)_2SO_4$ precipitate	1913	26	1.7
(3) Sephacryl S300 HR	36610	22	33
(4) Mono-Q peak 2	203309	1.92	181

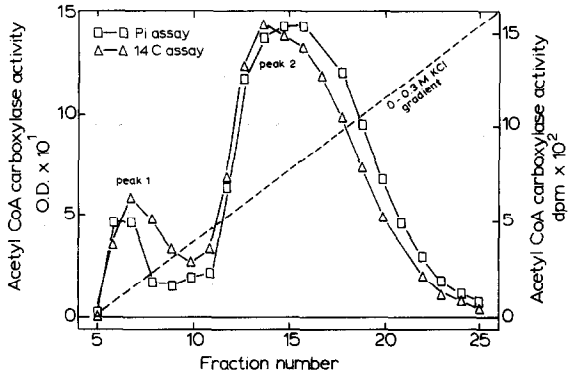

Fig.1. Purification of maize acetyl-CoA carboxylase using Mono-Q, measured using ^{14}C and phosphate assays.

3. RESULTS

The activity of acetyl-CoA carboxylase during purification from maize shoots was followed using the ^{14}C assay described. This gave a measure of the overall purification factor (table 1). After elution from the Mono-Q column, two peaks of enzyme activity were seen with the ^{14}C assay. We were able to establish that acetyl-CoA carboxylase activity could also be determined by measuring the liberation of inorganic phosphate in the presence of acetyl-CoA, and two peaks of activity were confirmed by this procedure as well. With both assays, activity was localized in the same fractions (fig.1), and measurement with the phosphate assay gave a specific activity in fraction 15 of 173 nmol/min/mg. The first peak of acetyl-CoA carboxylase activity (peak 1) coincided with a major protein peak which also contained non-specific phosphatases (seen as an increase in phosphate production in the absence of acetyl CoA).

Analysis of the Michaelis-Menten kinetics for acetyl-CoA carboxylase activity in peak 2 with respect to acetyl-CoA gave K_m values of 0.236 mM (±0.060, n = 4) for the phosphate assay, and 0.279 mM (±0.065, n = 3) for the ^{14}C assay. Kinetics for inhibition by fluazifop were also investigated with both assays using acetyl-CoA carboxylase activity in peak 2 and acetyl-CoA as the variable substrate (figs 2 and 3); in both experiments inhibition was non-competitive.

Table 2

Inhibition of peak 1 and 2 maize acetyl-CoA carboxylase by avidin

	Phosphate assay	^{14}C assay
Peak 1 (0.2 units avidin/sample)	83%	78%
Peak 2 (2 units avidin/sample)	91%	86%

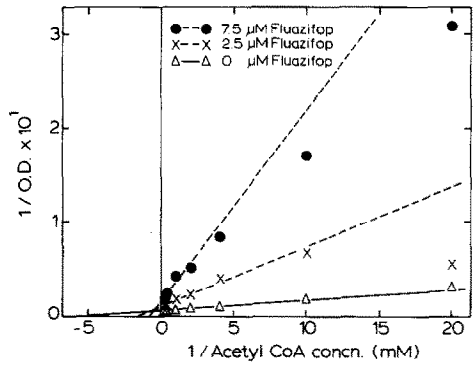

Fig.2. Inhibition kinetics for fluazifop acid, with acetyl-CoA as the variable substrate, measured using the P_i assay.

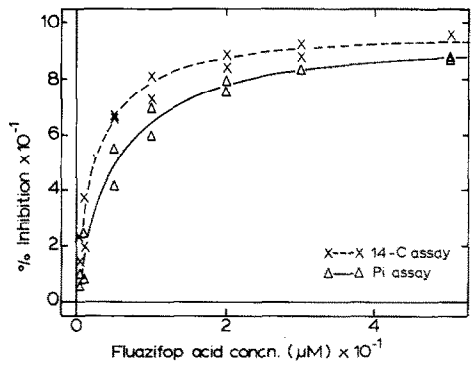

Fig.4. IC_{50} for fluazifop acid measured using ^{14}C and phosphate assays.

No measurable inhibition of peak 1 activity by fluazifop was found at doses up to 50 μM using both assays, but in preliminary experiments with the biotin antagonist, avidin, both peaks 1 and 2 were inhibited, and similar results were obtained with both the phosphate and the ^{14}C assay. The results are shown in table 2.

Both assays showed inhibition of peak 2 acetyl-CoA carboxylase by this compound, and dose-response curves using both assays gave similar IC_{50} values (peak 2 IC_{50}, phosphate assay: 4.99 μM; ^{14}C assay: 2.45 μM) (fig.4).

4. DISCUSSION

With the recent increased interest in acetyl-CoA carboxylase from plant sources as the site of action of new grass-selective herbicides, there is a need for a more readily accessible assay procedure to accommodate kinetic analyses, and this paper addresses that problem. Acetyl-CoA carboxylase has been partially purified from maize in a 3-step process providing a 180-fold purification. In comparing enzyme activity assayed by ^{14}C fixation, and by phosphate release, it could be clearly seen in fig.1 that there is a close correlation between the two procedures for the activity of acetyl-CoA carboxylase eluting from an ion-exchange column. Both methods registered major (peak 2) and minor (peak 1) peaks of activity with the purer product being in peak 2.

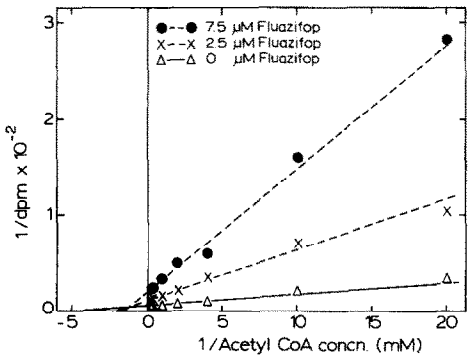

Fig.3. Inhibition kinetics for fluazifop acid, with acetyl-CoA as the variable substrate, measured using the ^{14}C assay.

Since phosphate is liberated as a product of the first half of the reaction sequence, the two reactions must remain tightly coupled during purification, and both reactions must be present in the two peaks of activity. Measurement of K_m values for acetyl-CoA by the two assay procedures using the activity in peak 2 gives closely similar results. The values are also similar to those reported in maize [16], spinach and avocado [12], and wheat germ [17].

Inhibition kinetics by the two assays both indicate non-competitive inhibition of acetyl-CoA carboxylase in peak 2, and the IC_{50} values at 3–5 μM show no significant difference; obtaining accurate K_i values awaits kinetic analyses that are more extensive than reported here. It is therefore clear that the total reactions that constitute activity of acetyl-CoA carboxylase can be recorded reliably using the liberation of phosphate in the first half of the reaction sequence because the two parts of the sequence remain tightly coupled. Furthermore, because of the non-specific absorption of CO_2 in the reaction due to the partial pressure of carbon dioxide in the atmosphere, it may be anticipated that the specific activity of the enzyme measured by phosphate release is more accurate than that measured by ^{14}C fixation. The use of the phosphate assay provides advantages over the ^{14}C assay in the speed of analysis and in accuracy, though the former suffers in crude plant extracts where high levels of non-specific phosphatase activity may be present.

The acetyl-CoA carboxylase activity in peak 1 is not inhibited by fluazifop although it is inhibited by avidin. This raises questions about the nature of the peak in terms of aggregates or isoforms, and will be the subject of a future publication.

REFERENCES

[1] Burton, J.D., Gronwald, J.W., Somers, D.A., Connelly, J.A., Gengenbach, B.G. and Wyse, D.L. (1987) Biochem. Biophys. Res. Commun. 148, 1039–1044.

[2] Secor, J. and Cseke, C. (1988) Plant Physiol. 86, 10–12.

[3] Kobek, K., Focke, M. and Lichtenthaler, H.M. (1988) Z. Naturforsch. 43c, 47–54.

[4] Rendina, A.R., Felts, J.M., Beaudoin, J.D., Craig-Kennard, A.C., Look, L.L., Paraskos, S.L. and Hagenah, J.A. (1988) Arch. Biochem. Biophys. 265, 219–225.

[5] Walker, K.A., Ridley, S.M., Lewis, T. and Harwood, J.L. (1988) Biochem. J. 254, 307–310.

[6] Focke, M. and Lichtenthaler, H.K. (1987) Z. Naturforsch. 42c, 1361–1363.

[7] Rendina, A.R. and Felts, J.M. (1988) Plant Physiol. 86, 983–986.

[8] Wood, H.G. and Barden, R.E. (1977) Annu. Rev. Biochem. 46, 385–413.

[9] Ryder, E., Gregolin, C., Chang, H. and Lane, M.D. (1967) Proc. Natl. Acad. Sci. USA 57, 1455–1462.

[10] Hellyer, A., Bambridge, H.E. and Slabas, A.R. (1986) Biochem. Soc. Trans. 14, 565–568.

[11] Finlayson, S.A. and Dennis, D.T. (1983) Arch. Biochem. Biophys. 225, 586–595.

[12] Mohan, S.B. and Kekwick, G.O. (1980) Biochem. J. 187, 667–676.

[13] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.

[14] Nikolau, B.J., Hawke, J.C. and Slack, C.R. (1981) Arch. Biochem. Biophys. 211, 605–612.

[15] Hess, H.H. and Derr, J.E. (1975) Anal. Biochem. 63, 607–613.

[16] Nikolau, B.J. and Hawke, J.C. (1984) Arch. Biochem. Biophys. 228, 86–96.

[17] Mishina, M., Kamiryo, T., Tanaka, A., Fukui, S. and Numa, S. (1976) Eur. J. Biochem. 71, 295–300.