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Biochemical evidence for multiple acetoin-forming enzymes in cultured plant cells☆

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Abstract

Acetoin (3-hydroxy-2-butanon) production was investigated in extracts from suspension cultured cells of carrot, tobacco, maize and rice. Crude extracts were able to catalyze acetoin synthesis from pyruvate and/or acetaldehyde at rates ranging from 0.02 to 0.1 mkat kg^{-1} protein, while no evidence was found for acetolactate-deriving acetoin production. Three acetoin-forming enzymes were resolved upon adsorption chromatography. A minor peak of activity was deduced as due to a partial, non-enzymatic decarboxylation of the acetolactate produced by acetolactate synthase under the same experimental conditions, being completely abolished by the addition of an acetolactate synthase inhibitor. The other two activities were characterized following further purification by gel filtration chromatography. A low molar ratio between acetoin production and pyruvate utilization, the capability of producing acetaldehyde from pyruvate at higher rate, an optimal activity at acidic pH values and its increase in extracts from cells grown under hypoxic condition strongly suggested the former as a side reaction of pyruvate decarboxylase. The latter activity, which showed maximal rate at neutral pH values, was on the contrary found to quantitatively convert acetaldehyde and pyruvate to acetoin. This pyruvate carboligase, which increased in actively proliferating cells and declined in a late logarithmic phase and was not induced under anaerobiosis, was present at similar levels in all four plant species.

Keywords: *Daucus carota*; *Nicotiana plumbaginifolia*; *Oryza sativa*; *Zea mays*; Acetoin biosynthesis; Acetolactate synthase; Acetolactate decarboxylase; Pyruvate carboligase; Pyruvate decarboxylase; Side reaction

1. Introduction

Acetoin (3-hydroxy-2-butanon) is a metabolite whose production has been recognized in bacteria as a mechanism for diverting glucose catabolism from acidic to neutral products (Booth, 1985; Tsau, Guffanti, & Montville, 1992). Under low pH and oxygen-limiting conditions, microbial cells which have entered the stationary phase of growth channel pyruvate into the 2,3-butanediol pathway (Magee & Kosaric, 1987) (Fig. 1): a catabolic acetolactate synthase (ALS, EC 4.1.3.18) catalyzes the condensation of two pyruvate molecules into α-acetolactate, which is decarboxylated to acetoin by an acetolactate decarboxylase (ALDC, EC 4.1.1.5). The latter is either excreted into the medium (Zahler, Najimudin, Kessler, & Vandeyar, 1988), or reduced to 2,3-butanediol possibly contributing to the regulation of the $NADH/NAD^+$ ratio during fermentative metabolism (Gotfredsen, Lorck, & Sigsgaard, 1983). Moreover, acetoin may derive also as a side product of most thiamine pyrophosphate (TPP) dependent pyruvate-decarboxylating enzymes, via the formation of the intermediate hydroxyethyl-TPP and the condensation of the latter with an acetaldehyde moiety (the so termed *carboligase reaction*; Fig. 1). However, even if in-vitro acetoin formation has been well established in the case of yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) (Chen & Jordan, 1984), and bacterial PDC (Juni, 1961), pyruvate oxidases (EC 1.2.2.2 and 1.2.3.3) (Bertagnolli & Hager, 1993) and pyruvate dehydrogenase (EC 1.2.4.1) (Hillman, Andrews, & Dzuback, 1987), its actual occurrence and physiological role in-vivo are still unclear.

☆ Part 1 in the series "Acetoin Synthesis in Higher Plants".

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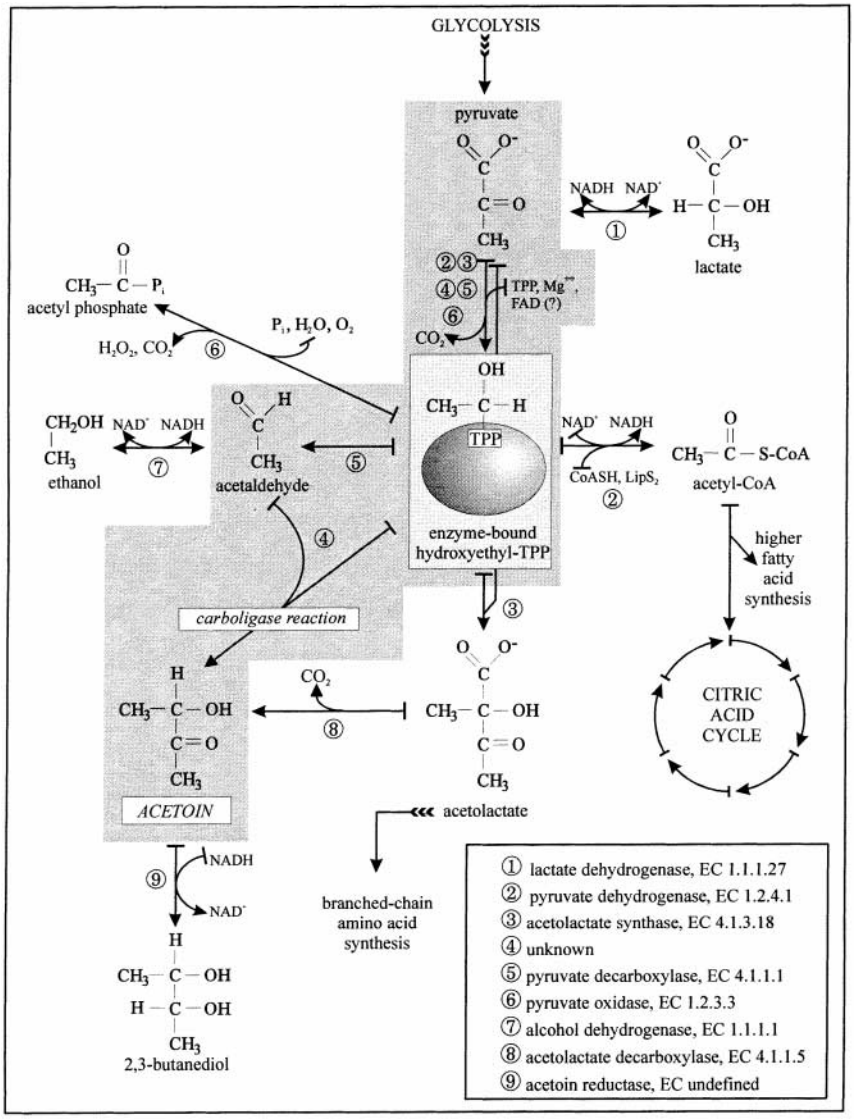

Fig. 1. Metabolic relationships of acetoin. It may derive from either the decarboxylation of acetolactate produced by acetolactate synthase, or the condensation of acetaldehyde with enzyme-bound hydroxyethyl-thiamine pyrophosphate (TPP), a reaction which seems to be catalyzed in vitro by most pyruvate-decarboxylating enzymes.

As regards plants, very little is known. A considerable synthesis of acetoin from pyruvate by crude extracts has been indeed widely reported in studies on anabolic ALS, the first enzyme of the path leading to branched-chain amino acid synthesis, which is measured indirectly after acid-decarboxylation of the acetolactate produced to acetoin (Bauerle, Freundlich, Störmer, & Umbarger, 1964) by a colorimetric titration of the latter (Westerfeld, 1945). Such direct acetoin production has been generally ignored, or interpreted as entirely due to an auxiliary reaction of PDC (Shimizu, Nakayama, Nakao, Nezu, & Abe, 1994), whose in-vitro carboligase activity was early shown in wheat germ (Singer & Pensky, 1952), and pea (Davies, 1964). PDC functions as a gateway leading from glycolysis to fermentation under hypoxic condition also in plants (Perata & Alpi, 1993), but a significance for acetoin production by PDC under anaerobiosis has not been investigated to date, nor the occurrence of the 2,3-butanediol pathway or other acetoin-producing enzymes.

Here we describe the detection and characterization in extracts from cultured plant cells of two different activities able to synthesize acetoin from pyruvate and/or acetaldehyde. According to previous reports one was deduced to be a side reaction of PDC, while the properties of the other were consistent with those expected for a specific pyruvate carboligase.

Table 1
Rates of acetoin synthesis from acetolactate, pyruvate and/or acetaldehyde in crude extracts from suspension cultured plant cells

	Specific activity (μkat kg^{-1})			
	D. carota	*N. plumbaginifolia*	*O. sativa*	*Z. mays*
Acetolactate (1 mM)	ND	ND	ND	ND
Pyruvate (20 mM)	83 ± 9	17 ± 6	150 ± 10	31 ± 8
Acetaldehyde (20 mM)	16 ± 2	4 ± 0	51 ± 7	4 ± 1
Pyruvate + acetaldehyde (10 mM each)	90 ± 9	29 ± 2	143 ± 10	41 ± 7
Pyruvate (2 mM)	17 ± 3	9 ± 0	9 ± 2	5 ± 2
Pyruvate + acetaldehyde (1 mM each)	13 ± 2	7 ± 1	5 ± 2	3 ± 1

Activities were calculated from linear regression of enzyme kinetics obtained by incubating 0.5 mg of protein up to 60 min in the presence of various substrate combination, as indicated. Values refer to extracts from cells harvested during the exponential phase of growth, and are mean ± SD of at least four independent determinations. ND, not detectable, <1 μkat kg^{-1}.

2. Results and discussion

2.1. Acetoin production in crude extracts

The occurrence of acetoin synthesis from acetolactate, pyruvate and/or acetaldehyde was investigated in extracts from actively-proliferating cells of carrot, tobacco, maize and rice. Results, summarized in Table 1, provided no evidence for ALDC activity; similar data were obtained with extracts from cells harvested either in the stationary phase of growth or following a 8 h treatment under hypoxic conditions (not shown) which in several microorganisms induces the expression of the 2,3-butanediol pathway (Magee & Kosaric, 1987). On the contrary, significant rates of acetoin synthesis were found with pyruvate as the substrate; acetaldehyde per se was also able to sustain acetoin production, even if at lower rates; the presence of both pyruvate and acetaldehyde enhanced low levels of acetoin synthesis (Table 1). Moreover, kinetics with pyruvate alone showed a pronounced lag, while with both substrates the reaction was found to be linear with time (not shown). When the substrate concentration was lowered to 1 mM, a dramatic decrease in acetoin production was evident (Table 1). Similar data may be consistent with the possibility that acetoin synthesis relies entirely upon a side reaction of PDC, as previously suggested (Singer & Pensky, 1952; Davies, 1964; Chen & Jordan, 1984; Shimizu et al., 1994): with pyruvate alone the reaction might not occur until significative levels of acetaldehyde have been produced, and at low substrate concentration pyruvate might be utilized almost completely before acetoin synthesis could take place. However, the concurrence of multiple pyruvate-consuming and/or acetoin-synthesizing activities in crude extracts might also lead to reciprocal interference, thus masking the presence of enzyme(s) specifically devoted to acetoin production.

2.2. Acetoin-forming activities following fractionation of crude extracts

When extracts of the four plant species were subjected to adsorption chromatography, three peaks of acetoin-synthesizing activities were indeed resolved (Fig. 2). A first, minor peak eluted at the same ionic strength at which ALS did. The addition to the reaction mixture of the herbicide chlorsulfuron at 10 μM, a concentration that causes a complete inhibition of ALS activity, abolished acetoin synthesis, too (not shown); the omission of glycerol from the extraction buffer, which strongly affects ALS recovery, resulted in a corresponding reduction of acetoin synthesis (data not presented). Thus acetoin production in peak 1 has most likely to be regarded as the result of a partial non-enzymatic decarboxylation of acetolactate produced by ALS which takes place under the mildly acidic assay condition employed.

A second, major acetoin-producing activity exactly matched the elution profile of PDC (Fig. 2). Since acetaldehyde synthesis in active fractions proceeded at a far higher rate, the occurrence of a side reaction which catalyzes the condensation of enzyme-bound hydroxyethyl-TPP with an already released acetaldehyde moiety, as previously shown for PDC of both microorganisms and plants (Singer & Pensky, 1952; Juni, 1961; Davies, 1964; Chen & Jordan, 1984), might easily account for the detection of lower levels of acetoin in the same fractions. However, a third enzyme able to produce acetoin from pyruvate and acetaldehyde was found which eluted at high values of ionic strength (Fig. 2): in this case active fractions were substantially devoid of other already known pyruvate-consuming activities, as PDC, pyruvate and lactate dehydrogenases, and pyruvate oxidase (data not shown).

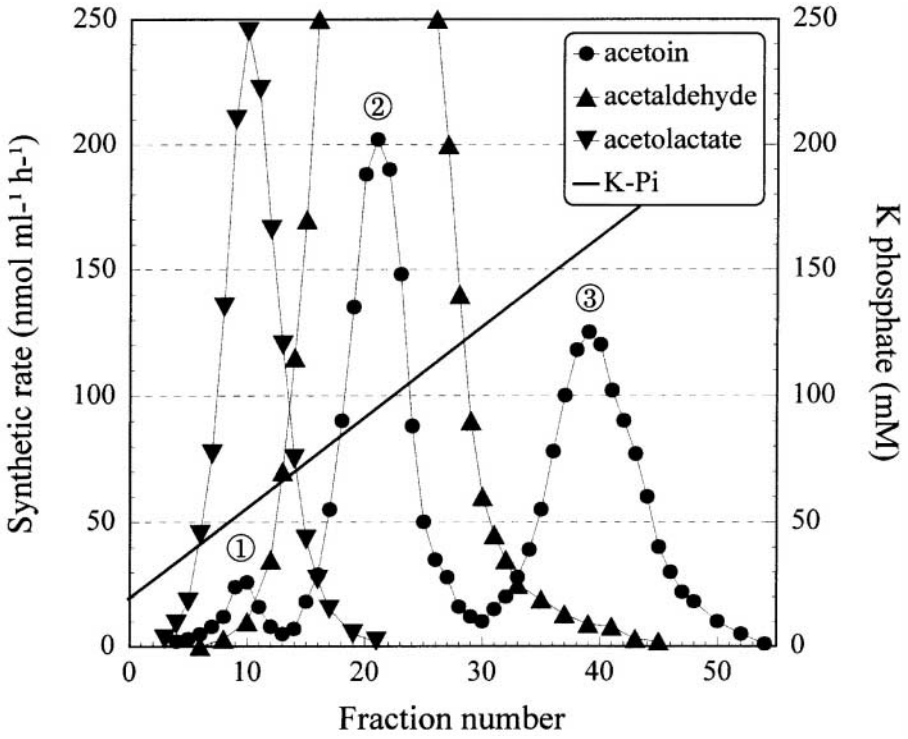

Fig. 2. Chromatographic separation of acetoin-forming activities. Crude extracts were subjected to adsorption chromatography on a hydroxyapatite column. Acetoin, acetolactate and acetaldehyde synthesis were measured in collected fractions in the presence of 5 mM $MgCl_2$ and 0.1 mM TPP, with 10 mM each pyruvate and acetaldehyde at pH 6.75, 40 mM pyruvate at pH 7.5, or 40 mM pyruvate at pH 5.75 as the substrate, respectively. Results refer to a typical experiment with extracts from *D. carota* cells, but similar data were obtained also with suspension cultured cells of tobacco, maize and rice.

2.3. Characterization of the two acetoin-forming enzymes

These two main activities were characterized following further purification by gel permeation chromatography. Activity in peak 2 eluted from a Sephacryl S300 column with a retention pattern consistent with a molecular mass of 250–280 kDa. Again, the elution profile was found to correspond exactly to that of PDC (not shown). Maize PDC was reported to be a very large protein, as it eluted immediately after the void volume from a Sepharose CL-6B column (Lee & Langston-Unkefer, 1985). However, this behavior might be caused by the tendency of the purified protein to aggregate under native conditions. Since reports of purification and sequence analyses of multiple PDC genes in maize accounted for two enzyme subunits of about 60–65 kDa (Lee & Langston-Unkefer, 1985; Kelley, 1989; Peschke & Sachs, 1993), our results may be consistent with a tetrameric native protein. On the contrary, activity in peak 3 showed retention volumes from a Sephacryl S200 column which suggests a native molecular mass of about 160 kDa (data not presented). Both activities strictly required TPP for catalysis (not shown). When the activity of the partially purified enzymes was assayed as a function of pH, the former exhibited maximal catalytic efficiency under mildly acidic conditions ranging from pH 5.5 to 6.7, and was sharply inhibited at values exceeding pH 7.0, while the latter retained maximal activity over a broad range from pH 6.0 to 8.0 (Fig. 3). Acidic pH optima have been indeed widely reported for PDC, which at the onset of anaerobiosis is not active: lactate production by lactic dehydrogenase in early phases of anaerobic metabolism results in a fall in cytoplasmic pH, and in the consequent activation of PDC, a pH-stat regulating the shift from lactic to ethanolic fermentative pathway (Perata & Alpi, 1993). Thus the acidic pH optimum for acetoin production in peak 2 further strengthens the hypothesis it is due to a side reaction of PDC. On the contrary, conversion efficiency experiments accounted for acetoin synthesis as the main reaction of activity in peak 3. When acetoin production and pyruvate consumption were measured at increasing time in the same reaction mixture, data were in fact consistent with half a mole of acetoin produced per mole of pyruvate utilized in the absence of acetaldehyde, and with a bit less than one mole of acetoin per mole of pyruvate in the presence of acetaldehyde (Fig. 4). The latter result most likely depends on the release of some hydroxyethyl moiety from the active site before its condensation into acetoin (Fig. 1)

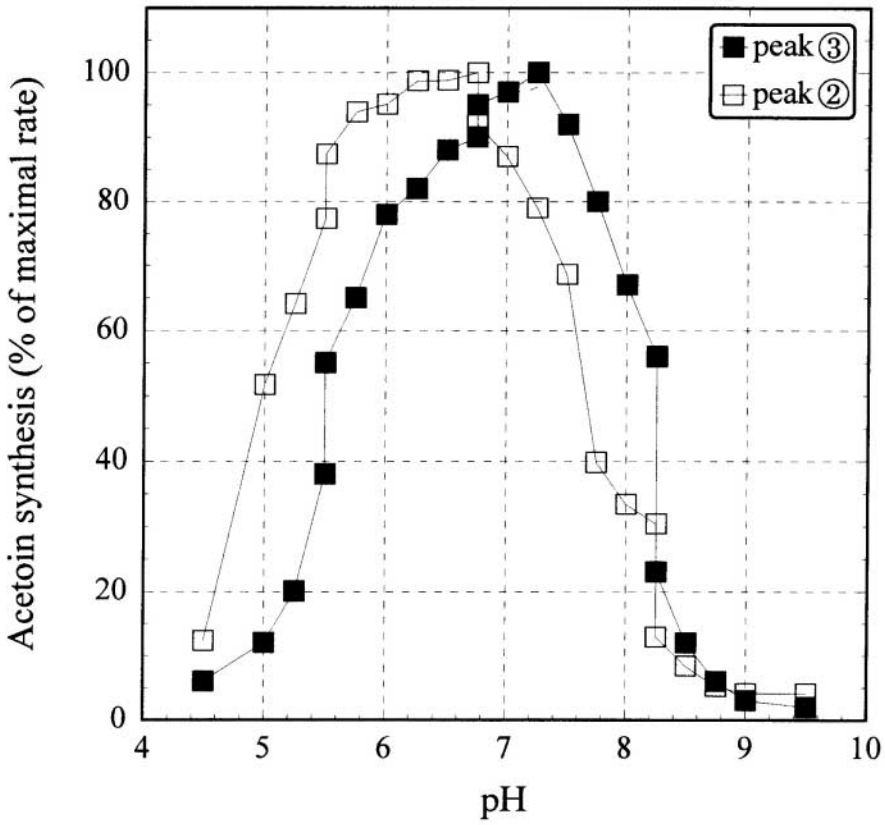

Fig. 3. pH–activity relationship of acetoin-synthesizing enzymes. Extracts from *N. plumbaginifolia* cells were fractionated by absorption chromatography; active fractions corresponding to peaks 2 and 3 in Fig. 2 were further processed by gel permeation, and the partially purified enzymes were assayed as a function of pH in the presence of acetate (pH 4.5–5.5), Mes (pH 5.5–6.75), Hepes (pH 6.75–8.25) and Tris (pH 8.25–9.5) buffer at 100 mM final concentration. The actual pH value of each sample was measured at the assay temperature with an electrode. Results were expressed as percent of maximal activity (about 0.98 and 1.15 mkat kg^{-1} of protein for activity in peak 2 and 3, respectively). Similar data were obtained with enzymes extracted from suspension cultured cells of carrot, maize and rice.

(Juni, 1961) even in the presence of exceeding acetaldehyde concentration. On a whole the above evidences suggest the occurrence in plant cells of a pyruvate carboligase specifically devoted to acetoin synthesis.

2.4. Differential expression of acetoin-forming activities

Specific activities of both enzymes were quantified in extracts from *N. plumbaginifolia* cells harvested at different stages during the growth cycle of the culture. Results showed that while acetoin production ascribable to PDC raised in the stationary phase, most likely due to the attendant establishment of hypoxic conditions, the putative pyruvate carboligase increased with the onset of exponential growth and declined in the stationary phase (Fig. 5). The expression at high rates in actively proliferating cells might thus indicate the involvement of the latter in some biosynthetic pathway. Specific activities were quantified also in exponentially growing cells of the other three cultures. Results (Table 2) showed that this enzyme is expressed at quite similar low levels even in distantly related plant species. When the cultures were subjected to a 8 h incubation under hypoxic conditions, according to previous reports (Kelley, 1989; Peschke & Sachs, 1993) PDC activity levels were found to be enhanced up to three-fold, while those of the pyruvate carboligase were not significantly affected (data not shown).

3. Conclusions

While no evidence was found for acetolactate-deriving acetoin production, significant rates of acetoin synthesis from pyruvate were detected in crude extracts from suspension cultured plant cells. Chromatographic fractionation of extracts allowed to detect in all four species tested two enzyme activities able to catalyze the condensation of an acetaldehyde moiety with protein-bound hydroxyethyl-TPP (Fig. 1). According to previous reports (Singer & Pensky, 1952; Juni, 1961; Davies, 1964; Chen & Jordan, 1984) biochemical properties and expression patterns strongly suggested the former as a side-reaction of PDC which takes place at saturating pyruvate concentrations. PDC activity was

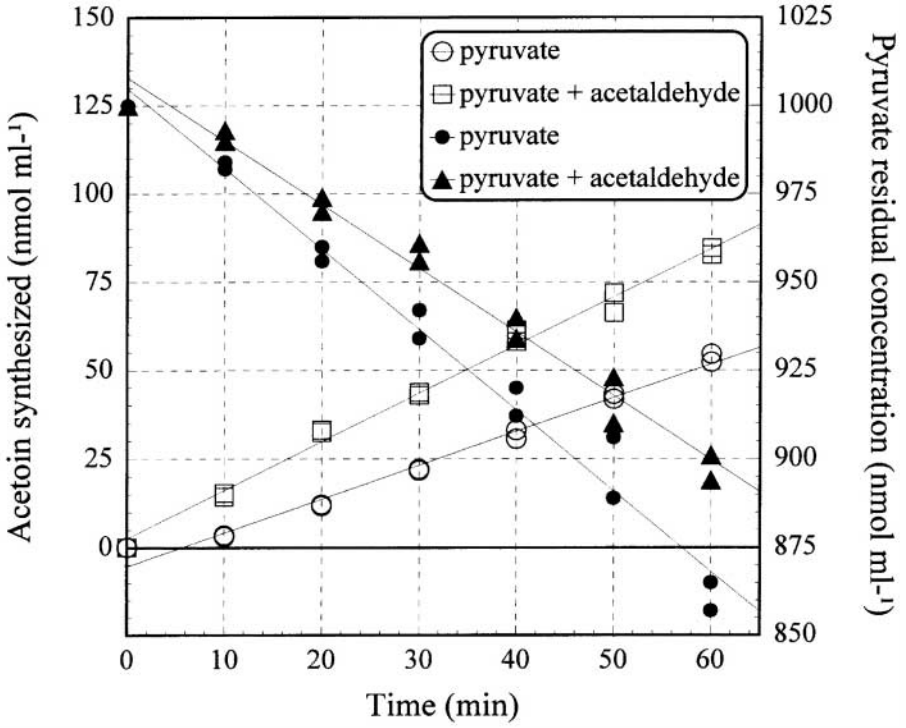

Fig. 4. Conversion efficiency of the putative pyruvate carboligase (peak 3 of Fig. 2) partially purified from *Z. mays* cultured cells. Acetoin production (open symbols) and pyruvate consumption (close symbols) were measured at increasing time by withdrawing aliquots from the same reaction mixture (10 ml) either in the presence or in the absence of acetaldehyde. In order to allow an accurate determination of residual pyruvate levels by the lactate dehydrogenase coupled assay, the concentration of both substrates was lowered to 1 mM. Under these conditions, acetoin synthesis proceeded at a rate corresponding to 76% of the value obtained with the same preparation at saturating substrate level.

found in fact to co-elute in both purification steps. In the absence of a 2,3-butanediol pathway, such acetoin production does hardly find a physiological significance, and may be simply interpreted as a *sponger* reaction, which could be artifactually enhanced in vitro by very high substrate levels. On the contrary, acetoin production seems to represent the main reaction of the latter enzyme, which exhibited maximal activity at neutral pH and higher expression in actively proliferating cells. These data, along with the apparent widespread presence in higher plants even if at very low levels, may account for a role in some biosynthetic route. A relation has indeed been hypothesized between acetoin synthesis and flavinogenesis, because of acetoin overproduction in a riboflavin deficient mutant of yeast (Nakajima & Saito, 1987). Work is currently under way in order to elucidate this aspect.

4. Experimental

4.1. Plant material

Cell suspension cultures of carrot (*Daucus carota* L. cv Lunga di Amsterdam), tobacco (*Nicotiana plumbaginifolia* Viviani), rice (*Oryza sativa* L. cv Roncarolo) and maize (*Zea mays* L. cv Black Mexican Sweet) were grown in MS medium (Murashige & Skoog, 1962) containing 0.5 mg l^{-1} 2.4D and 0.25 mg l^{-1} kinetin, 0.5 mg l^{-1} 2.4D and 0.5 mg l^{-1} 6-BAP, 2 mg l^{-1} 2.4D, or 1 mg l^{-1} 2.4D, respectively. Incubation was in the dark at 26 ± 1°C on a rotary shaker (120 rpm). Subcultures were made every two weeks by transferring 10 ml aliquots to 100 ml of fresh medium.

4.2. Chemicals

α-Acetolactate was prepared from ethyl-2-methyl-acetoacetate by the method of Krampitz (1957). Analytical grade chlorsulfuron (2-chloro-*N*-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino carbonyl]benzene sulfonamide) was a gift from E.I. DuPont de Nemours.

4.3. Preparation of crude extracts

Cells were harvested by vacuum filtration, powdered in liquid nitrogen and resuspended in 2 ml g^{-1} of extraction buffer (50 mM K phosphate, 5% v/v glycerol, 1 mM $MgCl_2$, 0.25 mM DTT and 0.1 mM TPP, pH 7.5) with 1% w/v insoluble PVPP added in order to prevent oxidation of phenolic compounds. All subsequent operations were carried out at 0–4°C. The homogenate was centrifuged at 20,000*g* for 30 min,

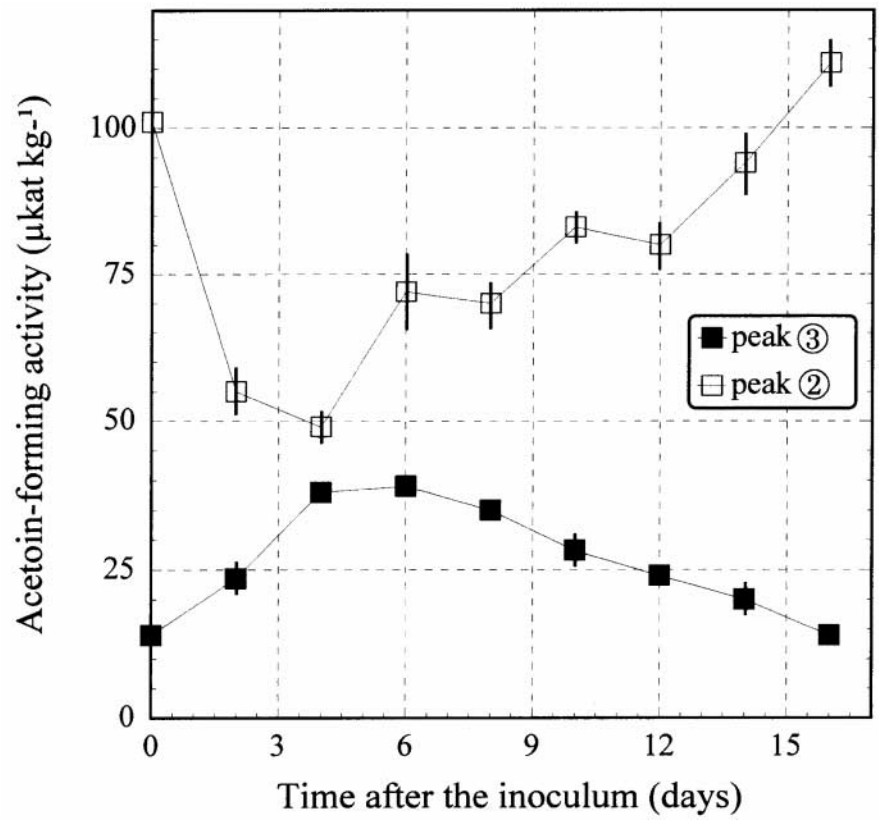

Fig. 5. Specific activity of acetoin-producing enzymes during the growth cycle of *N. plumbaginifolia* cultured cells. Values refer to the activities calculated from total recovery in the eluate following adsorption chromatography (Fig. 2) as related to the amount of protein layered onto column, and are mean ± SD of two independent experiments. Similar data were obtained also with suspension cultured cells of carrot and maize.

and the resulting supernatant was added with ammonium sulfate (70% of saturation). Proteins were collected by centrifugation at 12,000*g* for 20 min, resuspended in extraction buffer and desalted by passage through a Bio-Gel P6DG column.

Table 2
Specific activities of acetoin-forming enzymes in cultured plant cells

Plant species	Specific activity (μkat kg^{-1})	
	pyruvate carboligase (peak 3)	PDC (peak 2)
Daucus carota	44.1 ± 3.8	68.3 ± 23.4
Nicotiana plumbaginifolia	38.5 ± 2.8	54.9 ± 27.0
Oryza sativa	45.5 ± 11.4	94.3 ± 18.0
Zea mays	40.4 ± 4.5	52.2 ± 12.6

Activities were calculated from total recovery in the eluate following adsorption chromatography as related to the amount of protein layered onto the column. Values refer to extracts from cells harvested in the exponential phase of growth, and are mean ± SD of at least four independent determinations. PDC activity levels refer to the side, acetoin-synthesizing reaction, and range from 1.9 to 2.4% of the values obtainable by assaying the main, acetaldehyde-yielding reaction.

4.4. Enzyme assays

Except when indicated otherwise, acetoin synthesis was measured in a reaction mixture consisting of 50 mM MES–NaOH buffer (pH 6.75) containing 10 mM Na-pyruvate, 10 mM acetaldehyde, 5 mM $MgCl_2$, 0.1 mM TPP and a limiting amount of enzyme in a final volume of 0.4 ml. After incubation up to 60 min at 35°C, acetoin produced was quantified by the α-naphthol/creatine method (Westerfeld, 1945). Acetoin production from acetolactate (ALDC assay) was measured in the presence of 20 mM K phosphate buffer pH 7.5 by incubating enzyme with 1 mM freshly hydrolyzed methyl-acetoxy-ethyl-acetoacetate. Parallel assays were performed with heat-inactivated enzyme to quantify spontaneous acetolactate decarboxylation. Acetoin produced was determined as above. Acetaldehyde synthesis (PDC assay) was measured by a modification of the method of Ôba & Uritani (1982). A limiting amount of enzyme was incubated at 35°C in the presence of 50 mM MES–NaOH buffer (pH 5.75), 40 mM Na-pyruvate, 5 mM $MgCl_2$ and 0.1 mM TPP; at increasing times 0.1 ml aliquots were withdrawn and mixed with 0.9 ml of buffer containing 0.25 mM NADH and 0.17 μkat of yeast alcohol dehydrogenase, and after exactly 1 min at 35°C the resulting

decrease in absorbance at 340 nm was recorded. Acetolactate production (ALS assay) was measured as described previously (Forlani, Suardi, & Nielsen, 1996). Protein concentration was determined by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as a standard. Lactate dehydrogenase was assayed by continuously monitoring the decrease in absorbance at 340 nm at 35°C in the presence of 20 mM Na pyruvate, 1 mM $MgCl_2$ and 0.25 mM NADH. Pyruvate dehydrogenase and pyruvate oxidase activities were assayed as described (Camp & Randall, 1985; Bertagnolli & Hager, 1993). Pyruvate utilization in assay mixtures was determined by withdrawing 0.2 ml aliquots which were mixed with 0.8 ml of 50 mM K phosphate buffer pH 7.5 containing 0.5 mM NADH and 0.8 μkat of rabbit lactate dehydrogenase; after exactly 30 s at 35°C, the resulting decrease in absorbance at 340 nm was recorded.

4.5. Enzyme isolation

The 0–70% saturated ammonium sulfate fraction was column buffer-exchanged against 20 mM K phosphate extraction buffer and loaded onto a hydroxyapatite column equilibrated with the same buffer. After extensive washing, the column was eluted with a linear gradient from 20 to 200 mM K phosphate. Active fractions were pooled and concentrated by centrifugation in Ultrafree-CL filter units (Millipore; 100 kDa size exclusion). Two ml aliquots of the concentrated samples were layered onto a Sephacryl S200-SF (87×1.6 cm) or a Sephacryl S300-HR (85×1.6 cm) column that had been equilibrated with extraction buffer supplemented with 200 mM KCl, and eluted at a flow of 12 ml h^{-1}. Active fractions were desalted as above and stored until used at 0°C.

4.6. Hypoxic induction

A condition of low oxygen availability was achieved by simply incubating culture flasks without shaking. Such a treatment was preferred to an alternative procedure also tested, in which nitrogen was flushed into the culture, since the latter was found to rapidly lead to loss of cell viability (not shown).

Acknowledgements

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