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Purification and properties of a pyruvate carboligase from *Zea mays* cultured cells[1]

Giuseppe Forlani*

Department of Genetics and Microbiology, University of Pavia, I-27100 Pavia, Italy

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Abstract

An enzyme able to catalyze the synthesis of acetoin (3-hydroxy-2-butanon) from either pyruvate or acetaldehyde was isolated, partially purified and characterized from maize (*Zea mays* L. cv Black Mexican Sweet) cultured cells. It exhibited a maximal rate at neutral pH values, and strictly required thiamine pyrophosphate and a divalent cation for activity; on the contrary, unlike bacterial pyruvate oxidases, flavin was not required. Apparent Michaelis constants were 260 ± 20 μM for pyruvate and 24 ± 7 μM for acetaldehyde. Both substrate affinity and specificity were notably higher than those of pyruvate decarboxylase, an enzyme that also synthesizes acetoin as by-product. The partially purified protein was unable to catalyze the formation of other possible products of pyruvate decarboxylation, thus carboligase appears to be its main activity. Results suggest that acetoin synthesis may be of physiological significance in plants.

Keywords: Zea mays; Gramineae; Maize; Acetoin synthesis; Pyruvate carboligase; Pyruvate decarboxylase; Main/side reaction

1. Introduction

Under oxygen-limiting conditions, the high amount of pyruvate deriving from glycolysis may lead to cytoplasmic acidification. To achieve pH homeostasis, several microorganisms express a *catabolic* acetolactate synthase (ALS, EC 4.1.3.18) which channels pyruvate into the 2,3-butanediol pathway: the acetolactate produced is decarboxylated to a neutral compound, acetoin (3-hydroxy-2-butanon), that can be excreted or reduced in turn to 2,3-butanediol, possibly contributing also to the control of the NAD^+/NADH ratio (Magee & Kosaric, 1987). Even though contrasting evidence was earlier described (Bacher, Le Van, Keller, & Floss, 1983), acetoin has been hypothesized also as the 4-carbon compound needed for the formation of the *o*-xylene ring of riboflavin (Nakajima, 1985; Nakajima & Saito, 1987). In this case, acetoin synthesis should proceed independently from the expression of the 2,3-butanediol pathway. In fact, acetoin can derive also from the direct condensation of an acetaldehyde moiety with enzyme-bound hydroxyethyl-thiamine pyrophosphate (HE-TPP), which acts as intermediate in the two-site mechanism of pyruvate decarboxylation (Juni, 1961; Chen & Jordan, 1984). Indeed, the occurrence of a specific carboligase able to accomplish acetoin synthesis had been earlier hypothesized (Neuberg, 1946). However, increasing evidence that most TPP-dependent enzymes are capable of catalyzing this reaction (Chen & Jordan, 1984; Bertagnolli & Hager, 1993) led to a general consensus that, apart from the role in the inducible 2,3-butanediol pathway, acetoin may be a simple side-product of such a catalytic mechanism.

In plants, acetoin production from pyruvate in crude extracts has been widely reported in studies on *anabolic* ALS, that catalyzes the starting reaction in branched-chain amino acid synthesis. However, it has been ignored, or taken for granted as due to a side reaction of pyruvate decarboxylase (PDC, EC 4.1.1.1 (e.g. Shimizu, Nakayama, Nakao, Nezu, & Abe, 1994)). In fact, acetoin synthesis by PDC had been earlier shown in wheat germ (Singer & Pensky, 1952) and pea (Davies, 1964). However, when acetoin production was carefully investigated in cultured plant cells, results accounted for at least two different activities able to catalyze its synthesis. According to previous reports, one activity was found to rely upon a side reaction of PDC, while the properties of the other were similar to those expected for a specific pyruvate carboligase (Forlani, Mantelli, & Nielsen, in press). A thorough characterization of the latter enzyme,

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* Fax: +39-0382-528-496; E-mail forlani@ipvgen.unipv.it

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partially purified from cultured maize cells, is now reported.

2. Results and discussion

2.1. Purification of the putative pyruvate carboligase

Because of the presence of PDC and other pyruvate-utilizing enzymes, a quantitation of carboligase activity in crude extracts was not reliable. However, adsorption chromatography easily resolved it from most interfering enzymes, and allowed calculation of specific activities (Forlani et al., in press). The effect of the addition of various substances during the extraction was evaluated (Table 1). A protective effect was exerted by reducing compounds and cofactors (TPP and Mg^{++}) while, contrary to other pyruvate-decarboxylating enzymes (Chang & Cronan, 1988; Forlani, Riccardi, DeRossi, & De Felice, 1991), the presence of flavin adenine dinucleotide (FAD) did not improve recovery. A combination of adsorption, anion-exchange, negative affinity and gel filtration chromatography achieved an enrichment to a specific activity of 8 nkat mg^{-1}, thus reflecting a purification of over 200-fold, with a yield slightly exceeding 10% (Table 2). The relative lability of the enzyme, along with its very low level of expression, never allowed to obtain homogeneous preparations, as shown by SDS-PAGE analysis (not presented). Following gel filtration, more than 50% of activity was lost after 72 h storage at 0°C or after 24 h at −20°C; characterization experiments had thus to be performed soon after purification. The activity of the partially purified enzyme was linear over time only for a few dozen min. Then the rate of product increase declined, most likely being due to a gradual inactivation. However, the addition of 50 mM K–Pi (pH 6.75) to the assay mixture was found to exert a stabilizing effect and the rate was linear during up to 1 h at 35°C (data not shown). Seeing that enzyme preparations were not purified to homogeneity, in order to avoid artifacts in the subsequent characterization, proper checks were done to ensure that they were devoid of contamination by other pyruvate-consuming activities. ALS, lactate dehydrogenase (EC 1.1.1.27) and PDC were resolved early during the first step in the purification (not shown). The activity of pyruvate dehydrogenase (EC 1.2.4.1) was evaluated in pooled active fractions from the Sephacryl S200 column either in the presence or in absence of coenzyme A and cysteine, that of pyruvate oxidases (EC 1.2.2.2 and 1.2.3.3) following or not activation by SDS treatment, but in no case significant rates were found (Table 3).

2.2. Characterization of the partially purified enzyme

The enzyme was characterized with respect to kinetic and biochemical properties. As for the other pyruvate-decarboxylating enzymes, no activity was evident in the absence of TPP and divalent cations. A comparison was performed with the features of the acetoin-producing activity of PDC, that also was partially purified from maize cells to a specific activity of 3.9 nkat mg^{-1}, with a final enrichment of about 80-fold (data not reported). A remarkable difference was found with regard to the affinity for the substrates (Table 4). After an initial lag, both enzymes exhibited substantial activity with pyruvate alone and lower but significant rates with acetaldehyde alone (data not shown). This is consistent with previous data accounting for the reversibility of the reaction (HE-TPP↔acetaldehyde + TPP), that allows two acetaldehyde moieties to be condensed into acetoin even in the absence of pyruvate (Juni, 1961; Chen & Jordan, 1984). While PDC synthesized acetaldehyde at much

Table 1
Effect of the presence of various compounds during the extraction on the recovery of acetoin-synthesizing activities in extracts from *Zea mays* cells

	Specific activity (pkat mg^{-1})	
Extraction buffer	PDC	pyruvate carboligase
100 mM K–Pi, pH 7.5	19.6	31.5
100 mM K–Pi + 20% glycerol	46.0	15.6
100 mM K–Pi + 0.5 mM dithiotreitol	68.0	38.5
100 mM K–Pi + 0.1 mM TPP	36.0	43.6
100 mM K–Pi + 1 mM $MgCl_2$	31.6	41.1
100 mM K–Pi + 10 μM FAD	30.8	32.4
100 mM K–Pi with all the five additions	62.2	35.2

Cells in the exponential phase of growth were harvested and ground with liquid nitrogen. The powder was split into identical aliquots, which were resuspended in different buffers, as indicated. Activities were calculated from total recovery in the eluate following adsorption chromatography as related to the amount of protein layered onto column. Results refer to data obtained in a representative experiment. The rate of acetoin production by pyruvate carboligase was measured at pH 6.75, that of the by reaction of PDC at pH 5.75.

Table 2
Partial purification of *Zea mays* pyruvate carboligase

Step	Total activity (nkat)	Protein (mg)	Specific activity (nkat mg^{-1})	Purification (fold)	Yield (%)
0–70% ammonium sulfate	52.4	1,510	0.0347	1	100
Hydroxyapatite	32.1	182	0.176	5.1	61.3
DEAE-Sephacel	12.0	12.3	0.976	28	22.9
Blue-2 sepharose	10.1	5.5	1.84	53	19.3
Sephacryl S200	6.9	0.86	8.02	231	13.2

The results presented are the mean of three independent experiments, each starting from about 350 g (fr. wt) of cultured maize cells harvested in the mid-exponential phase of growth.

Table 3
Activity levels of other pyruvate-decarboxylating enzymes in a partially purified preparation of *Zea mays* pyruvate carboligase

Enzyme	Specific activity (nkat mg^{-1})
Pyruvate carboligase	7.78
PDC	0[a]
Lactate dehydrogenase	<0.3
ALS	<0.1
Pyruvate dehydrogenase	<0.3
Pyruvate oxidase, acetate-forming	<0.9
Pyruvate oxidase, acetyl phosphate-forming	<0.2

Aliquots of the partially purified enzyme corresponding to 25 pkat carboligase activity (about 3 μg of protein) were incubated up to 60 min under the proper assay conditions, as described in Section 3. Activities were thus calculated on the basis of the sensitivity of each assay method.

[a] PDC assay was performed by incubating the enzyme in the absence of ADH and withdrawing at increasing time aliquots which were mixed with buffer containing NADH and yeast ADH: results accounted for a release of acetaldehyde up to 10–15 μM during the first minutes of incubation, a level that remained substantially unchanged thereafter.

Table 4
Properties of *Zea mays* pyruvate carboligase compared to those of the acetoin-synthesizing activity of PDC

	Pyruvate carboligase	PDC
pH optimum[a]	6.7–7.0	5.6–5.9
$K_{m(app)}$ for pyruvate[b]	0.26 ± 0.02 mM	0.44 ± 0.03 mM
$K_{m(app)}$ for acetaldehyde[b]	24 ± 7 μM	5.1 ± 0.8 mM
$K_{m(app)}$ for TPP[b]	3.8 ± 0.3 μM	not determined
Molecular mass (by gel filtration)	158 ± 12 kDa	262 ± 30 kDa

[a] The pH optimum, evaluated in the presence of an equimolar mixture of MES–HEPES–AMPSO buffers (0.066 M each), is defined as the range in which more than 90% of maximal activity is exhibited.

[b] The nonvariable substrates were fixed at 5, 1 and 0.1 mM for pyruvate, acetaldehyde and TPP in the case of pyruvate carboligase, and 20, 50 and 0.1 mM in that of PDC, respectively.

higher rates than acetoin (about 520 versus 3.9 nkat mg^{-1}), in the presence of pyruvate alone the putative carboligase released low levels of acetaldehyde, up to 10–15 μM, only during the first minutes of incubation. This level did not increase thereafter (results not presented), most likely because acetaldehyde was condensed to acetoin as soon as it was produced. Consistently, the apparent Michaelis constant for acetaldehyde was in the micromolar range, whilst acetoin production by PDC was still enhanced at concentrations exceeding 10 mM

(Table 4, Chen & Jordan, 1984). Pyruvate-decarboxylating enzymes have been reported to use several other α-ketoacids (Bertagnolli & Hager, 1993), thus acetoin synthesis was measured with substrate of varying chain length. Results, summarized in Table 5, accounted for a higher substrate specificity of the putative carboligase: besides with pyruvate, it showed a significant catalytic rate only with the 4-carbon-chain compound α-ketobutyrate. On the contrary, PDC was found to utilize also 5- and 6-carbon-chain substrates. Finally, experiments were undertaken in order to ascertain a possible structural role for FAD. A number of enzymes, among which are the ALS family and bacterial glyoxylate carboligase, require flavin coenzyme for catalytic activity, even though the reactions catalyzed involve no redox reaction. This has been interpreted as a vestigial remnant of an ancient pyruvate oxidase from which such enzymes possibly evolved (Chang & Cronan, 1988). In the case of bacterial oxidases, that synthesize acetoin as by-product as well, FAD was found to be required for both the oxidative decarboxylation of pyruvate to acetate or acetyl phosphate and the nonoxidative carboligase reaction (Bertagnolli & Hager, 1993). The acid ammonium sulfate procedure developed to resolve FAD and apoenzyme (Gupta & Vennesland, 1964) was found to reduce the activity of a bacterial pyruvate oxidase to 24.9% of the untreated control; the subsequent addition of 10 μM FAD restored it up to 75.2% of the initial rate. On the contrary, specific activity of the partially purified pyruvate carboligase was reduced to 61.4% by the same treatment, but exogenous flavin coenzyme did not lead to any recovery of such activity loss.

2.3. Concluding remarks

Besides being the product of acetolactate decarboxylase (EC 4.1.1.5), acetoin has been reported to be synthesized as a side-product by most TPP-dependent decarboxylating enzymes (Chen & Jordan, 1984; Bertagnolli & Hager, 1993). Since there is no evidence for the occurrence of the 2,3-butanediol pathway in plants, the remarkable rates of acetoin synthesis from pyruvate in plant crude extracts have been interpreted as entirely due to such a by reaction of PDC (Shimizu et al., 1994). However, biochemical evidence was provided, accounting for at least one other enzyme able to catalyze substantial amounts of this compound (Forlani et al., in press). The properties of this enzyme, herein presented, are poorly consistent with those described for proteins that synthesize acetoin as a side product: low affinities for both substrates, mainly for acetaldehyde (Chen & Jordan, 1984) and low substrate specificity (Bertagnolli & Hager, 1993). In those cases acetoin formation was found to proceed much more slowly than the overall rate of pyruvate decarboxylation (Singer & Pensky, 1952; Juni, 1961; Chen & Jordan, 1984; Bertagnolli & Hager, 1993; this paper). Moreover, a structural requirement of FAD for the main activity resulted in the same requirement for acetoin synthesis, even though it does not involve any redox reaction (Bertagnolli & Hager, 1993). On the contrary, in the absence of acetaldehyde the enzyme partially purified from maize cultured cells produced as much as half a mol of acetoin per mol of pyruvate utilized (Forlani et al., in press). It showed both a high affinity and specificity of substrate and did not catalyze any of the other known reactions involving pyruvate decarboxylation. Carboligase seems thus to represent its main activity, a fact that would point at a physiological significance for acetoin also in plants. Therefore, it should be regarded not only as an artefactual product deriving from high substrate concentration in the assay mixture for ALS and PDC activity. As to a possible role for acetoin in plant cell

Table 5
Substrate specificity of *Zea mays* pyruvate carboligase

Substrate	Carbon chain length	Rate of acetoin production (% of maximal activity)	
		PDC	pyruvate carboligase
Glyoxylic acid	2	4.9	5.3
Sodium pyruvate	3	100.0	100.0
Sodium α-ketobutyrate	4	38.8	17.9
Sodium α-ketovalerate	5	29.3	3.5
Sodium α-ketoisovalerate	5	22.9	2.8
Sodium α-ketocaproate	6	14.8	3.6
α-Ketooctanoic acid	8	1.3	3.3

The rate of acetoin production by the partially purified enzymes was measured with α-ketoacids of varying chain length, each at a concentration of 5 mM. Acetaldehyde was added to an initial concentration of 1 and 10 mM for pyruvate carboligase and PDC, respectively. Results, expressed as percent of maximal activity, are averaged from two experiments performed using different enzyme preparations, with SD not exceeding 2%.

metabolism, it might be an intermediate in the biosynthetic route leading to the *o*-xylene ring of riboflavin, as hypothesized on the basis of incorporation experiments in the flavinogenic mold *Ashbya gossypii* (Nakajima & Saito, 1987). As an alternative, acetoin might represent the product of a detoxication path for acetaldehyde. Plant cells are very sensitive to the latter compound, for which an involvement in the induction of anoxia-related injuries has been suggested (Perata & Alpi, 1993). Because of the high affinity for acetaldehyde, the putative carboligase might rapidly convert it into the nontoxic acetoin. Recent findings seem to be consistent with this possibility (Otsuka, Mine, Ohuchi, & Ohmori, 1996). The availability of plant cell lines defective in this activity, currently under selection, might provide further information, and discriminate between these hypotheses.

3. Experimental

3.1. Plant material

Maize (*Z. mays* L. cv. Black Mexican Sweet) suspension cultured cells were grown in the dark as described previously (Forlani et al., in press).

3.2. Enzyme purification

All operations were performed at 0–4°C. Desalted crude extract in 75 mM K–Pi buffer containing 1 mM $MgCl_2$, 0.5 mM DTT and 0.1 mM TPP, prepared as described (Forlani et al., in press) from about 350 g (fr. wt) of cells harvested in the linear phase of growth, was added with solid ammonium sulfate up to 70% of saturation. Proteins were pelleted by centrifugation at $12{,}000 \times g$ for 20 min, resuspended in extraction buffer and desalted by passage through a Bio-Gel P6DG column. The desalted extract was loaded onto a hydroxyapatite column equilibrated with the same buffer. After extensive washing, enzyme activity was eluted with a linear gradient from 75 to 275 mM K–Pi. Active fractions were pooled and applied to a DEAE-Sephacel column equilibrated with 20 mM K–Pi extraction buffer. The column was eluted with a linear gradient from 0 to 400 mM KCl. Active fractions were pooled and passed through a Blue-2 sepharose column equilibrated with 20 mM K–Pi extraction buffer. The eluate was concentrated by filtration in a centrifugal ultrafree filter unit (100 kDa cut-off, Millipore). The concentrate, in a final volume of 2 ml, was loaded onto a Sephacryl S200 SF column (1.6×87 cm) equilibrated with extraction buffer containing 200 mM KCl. Elution proceeded at 12 ml h^{-1} while collecting 1-ml fractions. Pooled active fractions were column desalted as above against extraction buffer and stored at 0°C until used.

3.3. Enzyme assays

Except when indicated otherwise, acetoin synthesis by the partially purified pyruvate carboligase was measured in a reaction mixture consisting of 100 mM MES–NaOH buffer (pH 6.75) containing 10 mM Na-pyruvate, 10 mM acetaldehyde, 5 mM $MgCl_2$, 0.1 mM TPP, 50 mM K–Pi pH 6.75 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). Side-production of acetoin by PDC was assayed in a modified mixture in which K–Pi was omitted and pH was adjusted to 5.75. Acetaldehyde synthesis (PDC assay) was measured incubating the enzyme at 35°C in the presence of 50 mM MES–NaOH buffer (pH 5.75), 40 mM Na-pyruvate, 5 mM $MgCl_2$, 0.1 mM TPP, 0.25 mM NADH and 0.17 μkat of yeast alcohol dehydrogenase (ADH), continuously monitoring absorbance at 340 nm. As an alternative, enzyme was incubated in the absence of both NADH and ADH; at increasing times 0.2-ml aliquots were withdrawn and mixed with 0.8 ml of buffer containing NADH and ADH, and after 1 min at 35°C the resulting decrease in absorbance at 340 nm was recorded. The activity of acetolactate synthase, lactate dehydrogenase, pyruvate dehydrogenase and acetate- or acetyl phosphate-producing pyruvate oxidases was measured as indicated previously (Forlani et al., in press). Protein concentration was determined by the Coomassie Blue method (Bradford, 1976), using bovine serum albumin as a standard.

3.4. Preparation of apoenzymes and reconstitution with FAD

The enzyme partially purified from maize cells was subjected to the acid ammonium sulfate procedure developed by Gupta and Vennesland (1964). A bacterial pyruvate oxidase (Sigma P3673) was used as a positive control. Reconstitution was performed by allowing the apoenzyme to stand for 10 min in the presence of excess FAD (10 μM) at 35°C before starting the assay reaction.

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