Generation of Odorous Acyloins by Yeast Pyruvate Decarboxylases and Their Occurrence in Sherry and Soy Sauce

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Volatile acyloins (α-hydroxy ketones) were obtained by condensing either aldehydes with pyruvate or 2-keto acids with acetaldehyde in a reaction catalyzed by yeast pyruvate decarboxylases (EC 4.1.1.1). Odor qualities and threshold values of 34 acyloins were evaluated, and 23 of them possessed distinct flavor properties. Sherry and soy sauce flavors were analyzed: 2-hydroxy-3-pentanone and 3-hydroxy-2-pentanone were identified in soy sauce for the first time; these and 2-hydroxy-5-methyl-3-hexanone and 3-hydroxy-1-phenyl-2-butanone were isolated from sherry for the first time. The biocatalytic efficiencies of crude pyruvate decarboxylase preparations from *Zygosaccharomyces bisporus*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Kluyveromyces marxianus* were compared. Product yields comparable to those of conversions with purified pyruvate decarboxylase demonstrated the suitability of crude enzyme extracts as cost-effective biocatalysts in acyloin formation. Conversion rates of >50% showed that the potential of this type of enzyme to catalyze the formation of aliphatic acyloins has been underestimated before.

Keywords: *α-Hydroxy ketones; flavor compounds; pyruvate decarboxylase; Zygosaccharomyces bisporus*

INTRODUCTION

Pyruvate decarboxylase (PDC, E. C. 4.1.1.1) is a key enzyme in alcoholic fermentation and catalyzes the non-oxidative decarboxylation of pyruvate to acetaldehyde using thiamine diphosphate and Mg (II) ions as cofactors. The enzyme was first detected in yeast extracts by Neuberg and Karczag (1911) and occurs in many other fungi, plants, and bacteria. The formation of acyloins ("carboligation") as a side activity of PDC has been used since 1930 in the production of (*R*)-1-hydroxy-1-phenyl-propan-2-one (PAC, phenylacetyl carbinol) from benzaldehyde by yeast cells, serving as a chiral precursor for the synthesis of L-ephedrine. Proof that pyruvate decarboxylase is responsible for this biotransformation has been obtained much later by studying the isolated enzyme (Chen and Jordan, 1984; Bringer-Meyer and Sahm, 1988; Crout et al., 1991).

PDC condenses aldehydes and pyruvate to 3-hydroxy-2-oxo-acyloins, whereas 2-oxo acids and acetaldehyde give the isomeric 2-hydroxy-3-oxo-compounds. Both acyloins usually occur simultaneously, because a keto−enol equilibrium exists between them. Some secondary α-hydroxy ketones are well-known flavor components in food, such as the smallest representative of this class, acetoin (2-hydroxy-3-butanone). Besides acetoin, only eight other acyloins have been identified in food sources so far, five of them with reported flavor properties (Table 1). Acyloins as intermediates during the biosynthesis of solerone, one of the best known constituents of sherry aroma, are formed via PDC catalysis (Häring et al. 1997a,b).

In an earlier study, the formation of 34 aliphatic and aromatic acyloins by incubation of different aldehydes and 2-oxo acids with PDC from *Zygosaccharomyces bisporus* was reported (Neuser et al., 2000b). Most of them had not been known as natural products before. All of the volatile substances were now examined for their odor qualities and odor thresholds. The obtained GC retention indices and mass spectrometric data were employed to screen flavor extracts from fermented food for the occurrence of α-hydroxy ketones.

As the isolation and purification of pyruvate decarboxylase from yeast cells is a complex and time-consuming procedure (Neuser et al., 2000a), the applicability of crude enzyme extracts from different yeast strains to perform the respective biotransformation was evaluated.

MATERIALS AND METHODS

General. Thiamine diphosphate was purchased from Sigma Aldrich Chemical Co. All other chemicals were purchased from Fluka (Germany) and were of analytical grade quality.

Preparation of Crude Enzyme Extracts. The yeast strains *Zygosaccharomyces bisporus* CBS 702, *Kluyveromyces lactis* CBS 2359, and *Kluyveromyces marxianus* CBS 396 were obtained from the Centraalbureau voor Schimmelcultures (Baarn, Netherlands). Baker's yeast (*Saccharomyces cerevisiae*) was purchased from a local store. For the preparation of crude enzyme extracts, cultures were grown on a standard yeast medium containing 50 g L^{-1} glucose for 48 h. Cells were harvested by centrifugation and disintegrated in an agitated bead mill as described elsewhere (Neuser et al., 2000a). Cell fragments were separated by centrifugation and the clear supernatant was used as crude enzyme extract.

PDC Biotransformations. Pyruvate decarboxylase from *Zygosaccharomyces bisporus* was purified as reported elsewhere (Neuser et al., 2000a). PDC activity was measured at 25 °C by the coupled optical test according to Ullrich (1970). 5 U PDC (isolated or as crude enzyme extract) was mixed with 500 μM pyruvate, 100 μM aldehyde, and 0.1 M sodium citrate buffer (2 mM ThDP, 20 mM $MgSO_4$, pH 6.0) to give a final

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Table 1. Food Sources and Reported Flavor Properties of α-Hydroxy Ketones (Watanabe et al., 1988; Moio et al., 1993a,b; Burdock, 1995; Nijssen et al., 1996; Brock et al., 1984)

α-hydroxy ketone	natural occurrence in food	flavor properties
3-hydroxy-2-pentanone	cheese, durian, wine, asparagus, honey, tea, butter	herbaceous, truffle
2-hydroxy-3-pentanone	cheese, durian, coffee, wine, honey, butter	truffle, peanut
2-hydroxy-3-hexanone	wine	
4-hydroxy-3-hexanone	durian, tea	
3-hydroxy-5-methyl-2-hexanone	mozzarella cheese	melted cheese
3-hydroxy-2-octanone	beef and mutton fat (heated)	
5-hydroxy-4-octanone	cocoa	sweet, buttery, nut-like
3-hydroxy-4-phenyl-2-butanone	wine, sherry, honey	floral

Table 2. Odor Qualities and Threshold Values (in absolute ng of each odor impression, determined by Sniff-GC) of α-Hydroxy Ketones

α-hydroxy ketone	odor qualities	odor threshold (ng)
3-hydroxy-2-pentanone	caramel-sweet, buttery	500−600
2-hydroxy-3-pentanone	buttery, hay-like	250−300
2-hydroxy-3-hexanone	green, hay-like, sour milk	500−600
3-hydroxy-2-hexanone	earthy, mushroom-like	900−1000
2-hydroxy-3-heptanone	floral, buttery, mushroom-like	100−150
3-hydroxy-2-heptanone	earthy, hay-like, herbaceous	400−500
2-hydroxy-3-octanone	floral-sweet, buttery	40−50
3-hydroxy-2-octanone	earthy, mushroom-like, herbaceous	250−300
2-hydroxy-3-nonanone	buttery, sweet	100−120
3-hydroxy-2-nonanone	mushroom-like, buttery	500−600
2-hydroxy-3-decanone	fruity-sweet, floral, green	100−120
3-hydroxy-2-decanone	green, herbaceous, hay-like	700−800
2-hydroxy-5-methyl-3-hexanone	cheese, sour milk	400−500
3-hydroxy-5-methyl-2-hexanone	cheese, sour milk	800−900
2-hydroxy-4-methyl-3-heptanone	floral-green, hay-like	80−120
3-hydroxy-4-methyl-2-heptanone	floral-earthy, hay-like	200−250
2-hydroxy-4*E*-heptene-3-one	floral, spicy, earthy	50−80
3-hydroxy-4*E*-heptene-2-one	earthy, mushroom-like	250−300
2-hydroxy-4*E*-octene-3-one	floral, green, woody	20−30
3-hydroxy-4*E*-octene-2-one	earthy, floral, mushroom-like	80−100
2-hydroxy-5-(methylthio)-3-pentanone	spicy, meaty, sulfurous	15−20
3-hydroxy-5-(methylthio)-2-pentanone	spicy, alliaceous, sulfurous	5−10
3-hydroxy-4-phenyl-2-butanone	floral-sweet	75−100

volume of 4 mL. After 24 h at 24 °C, the mixture was extracted with diethyl ether (2 × 2 mL), and the extract was dried over anhydrous Na_2SO_4.

Gas Chromatography/Mass Spectrometry (GC/MS). A Fisons 8000 gas chromatograph equipped with an on-column injector (40 °C) was directly coupled with a Fisons MD 800 mass spectrometer. Separation of volatiles was achieved on a CW 20M fused silica column (30 m × 0.32 mm i.d., film thickness 0.25 μm, Macherey & Nagel, Germany), using helium as carrier gas (3.2 mL min^{-1}). The temperature program was 3 min isothermal at 40 °C, then raised to 230 °C at 3 °C/min. The temperature of the ion source was 200 °C, and the electron energy for all EI mass spectra was 70 eV.

Gas Chromatography/Olfactometry (GC/O). A Satochrom gas chromatograph was equipped with an on-column injector (40 °C) and a flame-ionization detector (FID, 230 °C). Column and temperature program were as described above and hydrogen was used as carrier gas. The gas flow was divided at a point 200 mm before reaching the detector. One part was led to the FID, and the other one was led to a heated sniff-port (200 °C). For the determination of odor properties, each enzymatically produced substance was isolated and concentrated by column chromatography on silica gel. Pure substances were sniffed in different intensities (concentrations) by a panel of at least six test persons. The odor threshold specifies the intensity at which at least 50% of the test persons were able to identify the substance by its characteristic odor quality.

Sherry and Soy Sauce Samples. Samples of two Spanish sherries (Sandemann Fino Dry and Amontillado Medium Dry) were purchased in a local store. Soy sauces included a Japanese (Kikkoman), a Chinese (Dark Soy Superior), and a Korean (Mong-Go) type which were obtained from an Asia market.

Isolation Procedures. The samples (500 mL) were diluted with 500 mL of KCl solution (2%) and extracted continuously with 350 mL of pentane/CH_2Cl_2 (2/1) overnight. The extract was dried over anhydrous Na_2SO_4 and concentrated using a Vigreux column at 40 °C.

RESULTS AND DISCUSSION

Odor Properties. We examined 34 enzymatically produced α-hydroxy ketones (17 pairs of isomers) by GC/olfactometry for their flavor properties. Odor thresholds were determined for compounds that showed interesting odor attributes. The flavor qualities of 19 out of the 23 substances listed had not been described so far (Table 2). Odor qualities were found to differ considerably within this class of odorants. The two components of an isomeric pair often showed similar sensory characteristics. The 2-hydroxy-3-oxo-acyloins displayed distinctly lower odor thresholds than the respective 3-hydroxy-2-oxo isomers, the methylthio-acyloins being the only exception in this respect and, moreover, the most powerful flavors examined. Eleven compounds were odorless under the conditions applied.

Biotechnological Production of Acyloins. The wide variety of odor properties observed turns α-hydroxy ketones into an interesting group of potential flavor chemicals. Biotechnological processes using purified enzymes as biocatalysts, however, may imply the disadvantage of high production costs. It was, therefore, examined whether crude yeast enzyme extracts show the same performance in acyloin formation as purified PDC. In addition to *Z. bisporus*, another two yeast strains (*Kluyveromyces lactis* and *Kluyveromyces marxianus*) and common baker's yeast (*Saccharomyces cerevisiae*) were tested. These strains were selected because

Table 3. Transformation of Hexanal or 2*E*-hexenal (25 mM) and Pyruvate (125 mM) by Crude Enzyme Extracts of Different Yeast Strains to 3-Hydroxy-2-octanone and 3-Hydroxy-4*E*-octene-2-one, Respectively

	3-hydroxy-2-octanone		3-hydroxy-4*E*-octene-2-one	
yeast strain	c (mM)	yield (%)	(mM)	yield (%)
Z. bisporus	9.62	39	5.98	24
S. cerevisiae	12.9	52	8.24	33
K. lactis	11.1	50	6.22	29
K. marxianus	12.5	44	7.25	25

sequence alignments showed high homologies of the PDC genes with that of *Z. bisporus* (Neuser et al., 2000a).

Hexanal and 2*E*-hexenal, abundant in many food flavors, were chosen as substrates for quantitative evaluation of the enzyme reaction. All of the four yeast species produced similar amounts of acyloins (Table 3). Baker's yeast, as the cheapest source of pyruvate decarboxylase, gave the highest product yields (52% for hexanal and 33% for 2*E*-hexenal, respectively). No significant differences in reaction rates were observed when compared to previous studies with purified PDC from *Z. bisporus*. The respective acyloins represented the main transformation products in all cases (> 95 area % in GC), which would facilitate product purification.

Natural Occurrence. The yeast PDC-catalyzed acyloin formation is a major biosynthetic pathway to α-hydroxy ketones. Two food sources which are traditionally obtained by yeast fermentation processes were, therefore, examined for the natural occurrence of acyloins. In addition to acetoin **1**, which is a well-known compound of wines (Nijssen et al., 1996), 3-hydroxy-2-pentanone **2**, 2-hydroxy-3-pentanone **3**, and 2-hydroxy-5-methyl-3-hexanone **4** were identified in both sherry samples (Table 4). Furthermore, 3-hydroxy-1-phenyl-2-butanone **5** and 3-hydroxy-4-phenyl-2-butanone **6** were detected in Dry Fino sherry (Figure 1, Table 1). The acyloins **2** to **6** occurred in small amounts only (<0.1 area %; ~10 to 100 ppb). Compounds **4** and **5** were detected as food components for the first time.

Out of three soy sauce samples examined, only the Japanese type showed a spectrum of volatile components typical of yeast-fermented products. Consequently, the α-hydroxy ketones **2** and **3** were detected for the first time along with acetoin in this sample (<0.1 area %; ~10 to 100 ppb) (Table 4).

The reported widespread occurrence of the two α-hydroxy pentanones in different food sources as well as in the here-examined sherry and soy sauce samples leads to the assumption that several biochemical pathways may exist apart from the one catalyzed by PDC. Alternative pathways have to be considered: the synthesis via 2-aceto-2-hydroxybutanoate, an intermediate in the formation of isoleucine, either enzymatically catalyzed by an acetolactate decarboxylase, or resulting from spontaneous disintegration of 2-aceto-2-hydroxybutanoate to 2,3-pentanedione and subsequent reduction (Figure 2) (Nykänen, 1986). The existence of pentanedione/hydroxy pentanone/pentanediol in wine as redox system, comparable to diacetyl/acetoin/butanediol has been discussed by Revel and Bertrand (1994). The actual origin of α-hydroxy pentanones in food could be further enlightened by chiral analysis, because the stereospecific action of PDC is known to result in *R*-acyloins (Kren et al., 1993; Lobell and Crout, 1996).

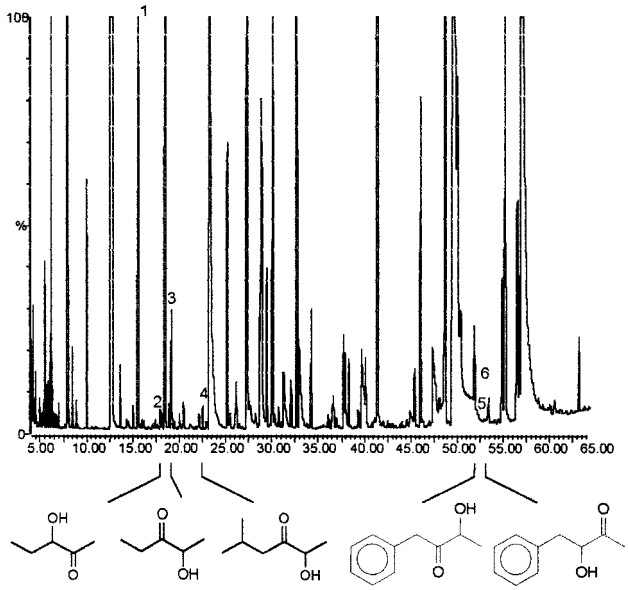

Figure 1. GC-MS chromatogram of sherry sample 1 (Fino Dry).

On the other hand, the close structural relationship of acyloin **4** to leucine and acyloins **5** and **6** to phenylalanine indicates their formation starting from the respective amino acids, a pathway proved by earlier qualitative and quantitative studies on the wild-type yeast *Zygosaccharomyces bisporus* (Neuser et al., 2000a, b). Amino acid precursors are either transaminated by transaminase or oxidized by l-amino acid oxidase to the corresponding 2-oxo acids which are further transformed by two PDC-catalyzed reaction steps (Figure 3). The limiting factor in acyloin formation in food matrixes might be the availability of 2-oxo acids and/or aldehydes as necessary precursors. Although in the case of the compounds **2**/**3** and **5**/**6** both tautomers were formed, the tautomer of **4** was not detectable in the aroma extract. Very likely, its concentration is below the detection limit or detection may be hampered by coelution of other volatiles. Because the C−C bond forming reaction represents a side activity of pyruvate decarboxylase only, rather high substrate concentrations are required for catalysis. An abundance of proteins and amino acids, respectively, as in the case of soy sauce, seems not to be crucial. The need for a specific regulation of metabolic processes as a result of elevated levels of ethanol might be of importance, as the different results for the sherry samples show. This is concluded from earlier attempts

Table 4. α-Hydroxy Ketones Identified in Sherry and Soy Sauce

α-hydroxy ketone	food source	KI[a]	*m*/*z*[b] (% relative abundance)
3-hydroxy-2-butanone **1**	sherry, soy sauce	1284	88 (21), 73 (5), 45 (100), 43 (88)
3-hydroxy-2-pentanone **2**	sherry, soy sauce	1346	116 (0.4), 73 (96), 55 (100), 45 (27), 43 (94)
2-hydroxy-3-pentanone **3**	sherry, soy sauce	1364	116 (1), 71 (28), 45 (100), 43 (84)
2-hydroxy-5-methyl-3-hexanone **4**	sherry	1453	130 (0.7), 85 (65), 57 (100), 45 (83), 43 (31)
3-hydroxy-1-phenyl-2-butanone **5**	sherry	2266	164 (1), 120 (21), 91 (100), 65 (33), 45 (53)
3-hydroxy-4-phenyl-2-butanone **6**	sherry	2257	164 (0.3), 121 (49), 103 (59),91 (100), 65 (32), 43 (46)

[a] Kovats index, CW 20 M. [b] Mass/charge ratio.

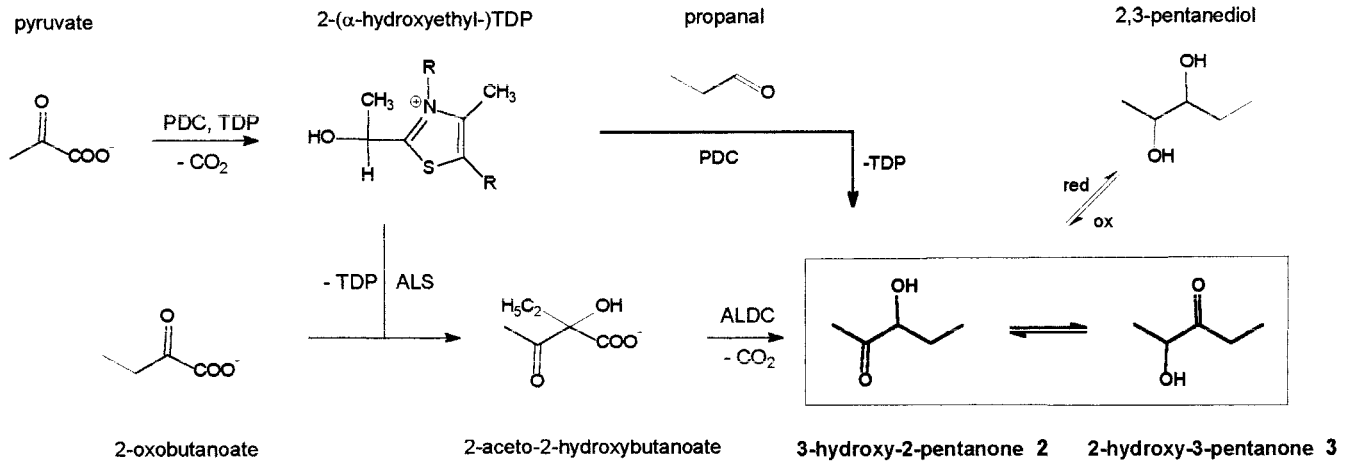

Figure 2. Formation of α-hydroxy pentanones (PDC, pyruvate decarboxylase; TDP, thiamine diphosphate; ALS, acetolactate synthase; ALDC, acetolactate decarboxylase).

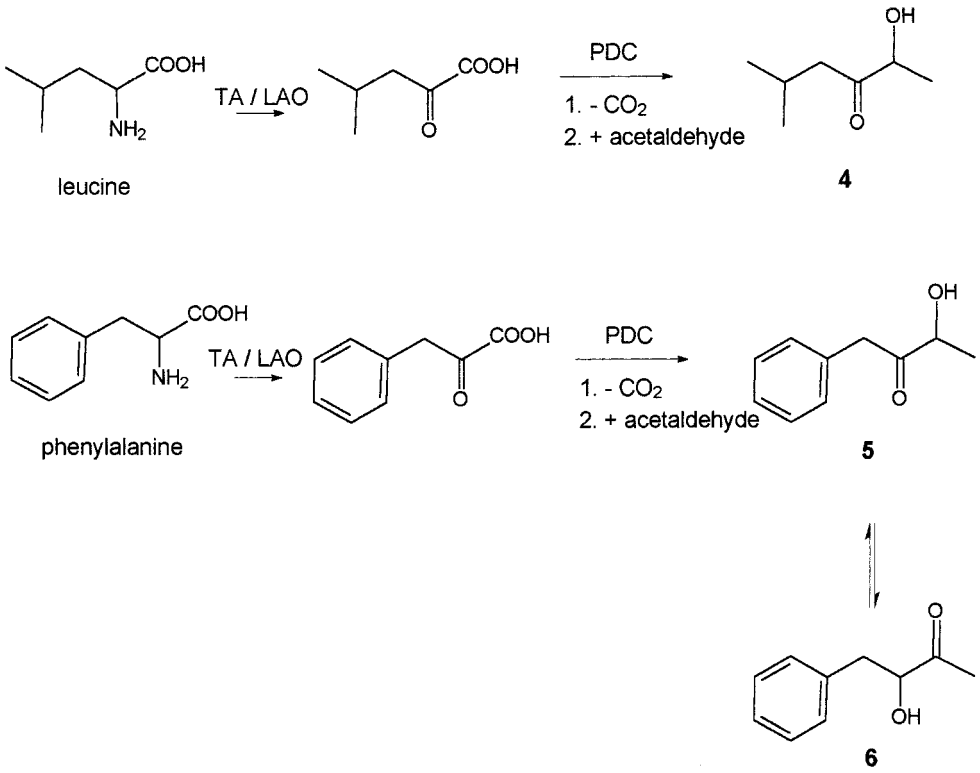

Figure 3. Formation of acyloins from amino acids by yeasts (TA, transaminase; PDC, pyruvate decarboxylase; LAO, L-amino acid oxidase).

of optimizing growth media for yeast cultures, when *Z. bisporus* produced the highest amounts of acyloins in the presence of 8% (v/v) ethanol (Neuser et al., 2000a).

CONCLUSIONS

The pleasant odor properties, along with low odor threshold values, of some of the α-hydroxy ketones presented might open possibilities for their future use as flavor ingredients in food. A recent patent application, describing the chemosynthesis of 3-hydroxy-5-methyl-2-hexanone and 2-hydroxy-5-methyl-3-hexanone indicates the high interest of the flavor industry (Gautschi and Ibanez, 1999). The application of crude enzyme extracts yielded amounts of acyloins comparable with the use of purified PDC from *Z. bisporus*. A cost-effective biotechnological production using a fixed bed enzyme reactor can be envisaged. Biotechnologically produced acyloins may be labeled as "natural" flavors and comply with the increasing preference of consumers for "natural" food

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