LETTER TO THE EDITOR

Δ- and E-Hydroxylation of Keto Acid by Mushrooms

Sir: The ability of actively fermenting yeast to reduce α- and β-keto acids to hydroxy acids has been known for sometime (1,2). More recently, it was demonstrated that a variety of yeasts, bacteria, and molds were capable of extending the reductive process to a series of alkyl monocarboxylic acids containing carbonyl functions at either the γ- or δ-positions of the carbon chain (3).

As it had been observed previously that higher fungi of the class Basidiomycetes could affect a variety of transformations on the steroid nucleus (4,5), it became of interest to see if these more highly differentiated organisms also contained enzymes which could affect changes in alkyl mono and dicarboxylic acids containing a carbonyl function located at various positions on the carbon chain.

Mushroom cultures for these studies were obtained from J.J. Ellis, Northern Utilization Research and Development Laboratories, Peoria, Ill. The following cultures were studied: NRRL 2366 *Pleurotus ostreatus*, NRRL 2367 *Collybia velutipes*, NRRL 2368 *Lepiota naucina*, NRRL 2369 *Morchella crassipes*, NRRL 2603 *Morchella esculenta*, NRRL 2370 *Cantharellus cibarius*, NRRL 2372 *Lycoperdon umbrinum*, and NRRL 2775 *Agaricus bisporus*. Stock cultures were maintained on 4% malt agar plates at 4 C. Mycelia for the bioreduction studies were obtained from dense mycelial mats grown in 2 liter Fernbach flasks containing 200 g glass beads and enough nutrient broth, plus 2% glucose, to cover the glass beads. Incubation at 25 C without agitation for 7-10 days produced a heavy mat. The mycelium was dispersed in a sterile blender (30 sec) and used at a 10-20% inoculum level.

Δ-keto-hexanoic acid was used in initial screening tests to determine which mushroom species could carry out the bioreduction process. Mycelium was inoculated into 250 ml Erlenmeyer flasks containing 50 ml nutrient broth and 2% glucose and incubated for 72 hr at 25 C on a rotary shaker. Cold-sterilized 5% δ-keto-hexanoate (1 ml) was added, and the incubation was continued for 30 min. An additional 1.0 ml substrate and 5.0 ml 30% sterile glucose then were added, and the fermentation was continued for 4 days. The medium was clarified by filtration, acidified to pH 2.0 with sulfuric acid, saturated with sodium chloride, and extracted with 50 ml diethyl ether (3x). The pooled ether fractions were washed with saturated sodium chloride (3x) and dried over anhydrous sodium sulfate crystals. The ether extracts then were evaporated to dryness with nitrogen.

The presence of δ-C_6-lactone was detected by its characteristic smell in fermentation extracts of four cultures. Strong aroma was produced by NRRL 2366, NRRL 2369, and NRRL 2603. Faint aroma was produced by NRRL 2370. No aroma was detected in NRRL 2367, NRRL 2368, NRRL 2372, and NRRL 2775 or in uninoculated keto-acid controls. Δ-C_6-lactone was purified further by dissolving the ether extract in 50 ml 1N potassium hydroxide and washing the caustic solution with 50 ml diethyl ether (3x). The potassium hydroxide solution was saturated with sodium chloride, acidified with sulfuric acid to pH 2.0, and reextracted into ether as before. Methyl esters of this acid fraction were prepared for definitive detection of keto- and hydroxy-acids.

Methylated fermentation extracts were analyzed on a 2 ft column of UCW-98 10%, held 2 min at 80 C, followed by temperature programing at 4 C/min to 200 C using a Beckman GC-5 gas chromatograph. Improved resolution of the lactone and keto-ester was obtained using a 6 ft., 20% Carbowax 20 M column, temperature programed at 4 C/min from 100-190 C. Peaks from the gas chromatogram were passed into a Hitachi RMU-6E single-focusing mass spectrometer operated at 70 eV using a 6 sec scan. Mass spectra confirmed the presence of δ-C_6-lactone and unreduced δ-keto-hexanoic acid.

The ability of *M. crassipes* NRRL 2369 to reduce various other medium chain keto-mono- and keto-dicarboxylic acids was investigated further. These fermentations, extractions, and analyses were conducted as previously described, except that 2 liter flasks containing 500 ml nutrient broth with 2% glucose were

TABLE I

Reduction of Keto-Acids by *Morchella crassipes* NRRL 2369 at 25 C for 96 hr

Keto acids	Mycelium dry wt (g)	Ether extract (mg)	Keto-acid (mg)	Hydroxy-acid (mg)	Lactone (mg)
Δ -C_5	1.80	160	56	—[a]	—
Δ -C_6	2.10	200	150	—	40
ϵ -C_7	2.20	233	70	11	—
α -C_8	0.60	172	—	—	—
α -C_9	0.70	68	25	5	—
α -C_7 (dioic)	1.80	48	13	—	—
Control	1.60	—	—	—	—

[a]— = No keto acid or reduction product detected.

used. Results of one experiment in which the sodium salts of keto-acids (0.5 g; except for 2-keto-nonanoic- 0.19 g) were added to actively metabolizing mushroom mycelium are presented in Table I. The formation of 6-hydroxy-heptanoic acid from its corresponding 6-keto-acid extends the specificity of the mushroom keto-reductase system to the ϵ- carbon in the chain. The inability of mushroom mycelia to reduce the keto function in 5-keto-valeric could be due to one of the following reasons: (A) an alkyl group on the omega side of the keto group may be necessary, (B) there may be a steric hindrance associated with the branched chain, or (C) an adjacent methylene group may be required. Interestingly, two of the keto compounds which can be reduced readily to the corresponding lactone (δ-C_6-lactone) or hydroxy-acid (6-hydroxy-heptanoic acid) stimulated mushroom growth, as evidence by increased mycelial wt. Conversely, both the C_8 and C_9 α-keto-acids may have been inhibitory to the mushrooms, as mycelial wt yields were reduced sharply in these flasks. Nevertheless, reduction to the hydroxy-acid readily occurred with the C_9-keto-acid substrate. The α-keto-C_5-dicarboxylic acid was not converted into a product; no hydroxy compound could be detected by gas chromatography.

Similar results to those described above were obtained when *P. ostreatus* NRRL 2366 was tested with the same substrates.

From the data provided it is evident that several mushroom species contain enzyme complements which enable them to reduce some alkyl-keto-acids to the corresponding hydroxy acids.

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