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α -Isopropylmalic Acid Accumulation by Leucine-requiring Yeast Mutants

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Since the discovery of α -isopropylmalic acid (β -carboxy- β -hydroxyisocaproic acid) as a precursor for the leucine biosynthesis in *Torulopsis utilis*,¹⁾ it has been reported by a number of investigators that the acid was produced as a metabolic product by some strains of bacteria and molds which require leucine for growth.^{2~5)} The occurrence of this acid in higher plants has also been reported.^{6~8)}

Recently, Strassman and Ceci⁹⁾ presented a detailed report on the enzymatic formation of α -isopropylmalic acid (α -isopropylmalate synthetase) in the cell-free preparations of baker's yeast. However, the accumulation of α -isopropylmalic acid as a metabolic product of leucine-requiring yeast mutants (auxotrophs) has not yet been reported.

During the courses of our study on citramalate formation by various yeast strains,^{10,11)} it was incidentally found that a considerable amount of α -isopropylmalic acid was accumulated in the culture medium of leucine-less mutants of *Saccharomyces cerevisiae*. The present paper briefly deals with the isolation and identification of α -isopropylmalic acid from the culture fluid of leucine-less yeast mutants.

A leucine-less mutant strain C1461-4D was statically grown for 10 days at 25°C in a chemically defined medium¹⁰⁾ supplemented with a small amount of L-leucine (10 mg/l of medium). The culture medium from which yeast cells were removed by centrifugation was acidified to pH 2.0 with sulfuric acid. The organic acids in the acidified medium were then extracted with ether for 72 hr using a liquid-liquid type extractor. The ether extract was then chromatographed on a silicic acid column (1.0×30 cm) as described previously,¹⁰⁾ and the chromatogram as shown in Fig. 1 was obtained. An acid corresponding to α -isopropylmalic acid was predominant, constituting 56% of the total amount of the acids produced by leucine-less mutant strain C1461-4D. Chromatography of the ether extracts was repeated to collect the acid fraction corresponding to α -isopropylmalic

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FIG. 1. Silicic Acid Column Chromatogram of the Organic Acids Produced by a Leucine-less Mutant Strain (C1461-4D) of *Saccharomyces cerevisiae*.

An ether-extract corresponding to 100 ml of culture fluid was applied.

FIG. 2. Infrared Spectra of Authentic DL- α -Isopropylmalic Acid and the Isolated Acids (in Nujol).

Curve A: Authentic DL- α -isopropylmalic acid.

Curve B: Acid produced by a mutant strain C1382-11D requiring both leucine and histidine.

Curve C: Acid produced by a mutant strain C1461-4D requiring leucine.

acid using a silicic acid column (1.6 $\times$ 30 cm). The acid fraction was evaporated to dryness under reduced pressure at 50–60°C, and the residue was extracted several times with hot ethyl acetate. The extract, decolorized with charcoal, was concentrated to a small volume to which ligroin was added to be a turbid solution. After allowed to stand for 2–3 hr at 5°C, the acid was obtained in a crystalline form. The acid was recrystallized to constant melting point by the same procedure.

The acid showed only one spot corresponding to authentic DL- α -isopropylmalic acid on paper chromatography, using four different solvent systems: R_f values were 0.85, (*n*-butanol-acetic acid-water, 52:14:34); 0.74, (phenol-formic acid-water, 75:1:25); 0.85, (*n*-butanol-formic acid-water, 4:1.5:1); 0.39, (cellosolve conc. ammonia-water, 80:5:15). The physical properties of the isolated acid were found to be: mp 169°C, $[\alpha]_D^{20}$ -19.6° ($c=5.0$ methanol), -19.6° ($c=2.5$ in water), -1255° in ($c=0.2$ in the presence of excess ammonium molybdate¹²). Found: C, 47.43; H, 6.86. Calcd. for C₇H₁₂O₅: C, 47.72; H, 6.87%. The infrared spectrum of the isolated acid was identical with that of authentic DL- α -isopropylmalic acid as shown in Fig. 2.

An acid corresponding to α -isopropylmalic acid, which was produced by a leucine and histidine-requiring mutant of *Saccharomyces cerevisiae* strain C1382-11D, was also separated and crystallized by the same procedure. The isolated acid showed mp 170°C, $[\alpha]_D^{20}$ -20.0° ($c=2.5$ in methanol), and an identical infrared spectrum to that of authentic DL- α -isopropylmalic acid (Fig. 2). Thus, it is evident that the acid accumulated by the two leucine-requiring mutants C1461-4D and C1382-11D was (–)- α -isopropylmalic acid.

Eight leucine-requiring strains of *Saccharomyces cerevisiae*, including multiple mutants requiring other amino acids and/or bases besides leucine, were then compared for the ability to accumulate α -isopropylmalic acid. In this experiment, each strain was statically grown for 6 days at 25°C in a 100-ml Erlenmeyer flask containing 50 ml of medium supplemented by the addition of leucine and other amino acids and/or bases (10 mg/l, each) according to the requirements of each strain. The culture fluids from which cells were removed by centrifugation were passed through a Dowex-1 $\times$ 2 column (acetate form, 1.0 $\times$ 30

¹² H. A. Krebs and L. V. Eggleston, *Biochem. J.*, **37**, 334 (1943).

cm) and the eluted fraction corresponding to α -isopropylmalic acid was iodometrically determined from the amount of methyl ketone compound formed upon permanganate oxidation. These procedures were essentially the same as those employed for citramalate determination in the previous paper¹¹⁾ except that 100 ml of the first eluate was discarded and 200 ml of the next eluate (2.5 N acetic acid) was collected. The methyl ketone compound, which is formed from the acid upon permanganate oxidation and which reacts with iodine in alkaline solution, was confirmed to be methyl isopropyl ketone from the retention time of gas-liquid chromatography. The retention time of this methyl ketone compound was identical with that formed from authentic

DL- α -isopropylmalic acid. As shown in Table I, all the eight leucine-requiring strains accumulated α -isopropylmalic acid in the culture media, though the amount of the acid varied from 13 to 75 mg per 100 ml of medium. On the other hand, it was found that none of the ten leucine-independent auxotrophic mutants (*Saccharomyces cerevisiae*) requiring other amino acids for growth could accumulate α -isopropylmalate in the medium. These facts clearly show that the α -isopropylmalic acid accumulation is correlated to the existence of a metabolic block in leucine biosynthesis. It seems that α -isopropylmalate synthetase, a condensing enzyme involved in the first step, is more active than the other intermediate enzymes of leucine biosynthesis under the leucine-insufficient condition.

The amount of α -isopropylmalic acid accumulated by the leucine-less mutant strain C1461-4D could be increased to 116 mg/100 ml of medium, when calcium carbonate (3%) was added to the medium. It was also found, however, that accumulation of α -isopropylmalate by the leucine-less mutant was almost completely inhibited by a higher concentration of L-leucine (500 mg/l of medium), indicating that the condensing enzyme (α -isopropylmalate synthetase) is also effectively regulated by end-product inhibition, a feed-back mechanism.

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TABLE I. ACCUMULATION OF α -ISOPROPYLMALIC ACID BY LEUCINE-REQUIRING MUTANT STRAINS OF *Saccharomyces cerevisiae*

Strain	Requirement	α -Isopropylmalic acid formed (mg/100 ml of medium)
C1461-4D*	leucine	74.7
C1461-10C*	leucine	64.2
C1462-5B	leucine	36.6
C1382-11D*	leucine and histidine	41.9
C814-45C*	leucine, histidine and uracil	32.6
C7-2A	leucine, lysine, methionine, tryptophan and adenine	13.6
S2583D	leucine, tryptophan, histidine, valine, isoleucine and adenine	24.4
S2582B	leucine, lysine, tryptophan, histidine, valine and isoleucine	38.4

The basal medium used was Yeast-Nitrogen-Base (Difco) plus glucose 5%; each of the amino acids and bases required was added at the concentration of 10 mg per litre of medium.

* These four mutant strains are presumed to be deficient in β -carboxy- α -hydroxyisocaproic acid dehydrogenase, based on the description of R.C. von Borstel¹³⁾ and the genetic analysis of C1461-4D.¹⁴⁾

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