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CONVERSION OF L-LEUCINE TO CERTAIN KETO ACIDS BY A TOMATO ENZYME PREPARATION

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Abstract—An enzyme preparation from fresh tomato fruit catalyzed the degradation of L-leucine. The reaction products were separated as their 2,4-dinitrophenylhydrazones and analyzed by TLC. α-Ketoisocaproic acid, the keto acid corresponding to L-leucine, was identified. The conversion was confirmed by studies with L-leucine-U-^{14}C as substrate. Enzyme activity was found to occur predominantly in the supernatant fraction and to decrease with ripening of fruit.

INTRODUCTION

LEONARD and Burris[1] demonstrated the presence and distribution of L-aspartate:2-ketoglutarate aminotransferase (EC No. 2.6.1.1) in several mature plant tissues, but could not detect the enzyme in green apples and green tomatoes. Hulme *et al.*[2] recently showed that the aminotransferase activity of mitochondria from apple fruit increased markedly during the climacteric, suggesting that this enhanced activity might serve as a regulatory mechanism in succinate oxidation by removal of the inhibitory oxaloacetate.

Yu *et al.*[3,4] reported that certain amino acids could serve as precursors of volatile components in tomato fruit, and suggested that the conversion might involve transamination. The production of the individual transamination products, however, was not shown. More recently[5] they demonstrated the production of 3-methylbutanol and 3-methyl-1-butanol from L-leucine by a crude tomato extract, and suggested that this amino acid might play an important role as a precursor of aroma components in tomatoes. If the conversion of L-leucine to these volatile components takes place through transamination, it should be possible to detect α-ketoisocaproic acid. The present study was initiated to test this possibility. By employing techniques different from those previously reported,[3–5] we have been able to demonstrate the presence of L-leucine:2-oxoglutarate aminotransferase (EC No. 2.6.1.6) in tomato fruit.

RESULTS

The absorption spectrum of the acidic 2,4-dinitrophenylhydrazone (DNPH) derivatives of the products from the reaction mixture containing added leucine was markedly different from that of the reaction mixture without added leucine. The spectrum had a maximum

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[1] M. J. K. LEONARD and R. H. BURRIS, *J. Biol. Chem.* **170**, 701 (1947).
[2] A. C. HULME, M. J. C. RHODES and L. S. C. WOOLTORTON, *Phytochem.* **6**, 1343 (1967).
[3] M. H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **7**, 555 (1968).
[4] M. H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **7**, 561 (1968).
[5] M. H. YU, D. K. SALUNKHE and L. E. OLSON, *Plant Cell Physiol. (Japan)* **9**, 633 (1968).

absorption peak at 440 and a minor peak near 540 nm. Standard α-ketoisocaproic acid, treated in the same way as the sample, showed a similar absorption spectrum.

TLC of the DNPH in benzene/acetic acid (19:1, v/v)[6] showed that two spots resulted from the presence of added leucine in the reaction mixture. They were identified as pyruvic acid–DNPH and α-ketoisocaproic acid–DNPH. Their R_f values corresponded to those of authentic samples. This was confirmed by TLC analyses in three other solvents (Table 1).

TABLE 1. TLC OF ACIDIC DNPH PRODUCTS RESULTING FROM L-LEUCINE CATABOLISM BY TOMATO ENZYME

	R_f of DNPH			
Solvent	Pyruvic acid	Unknown	α-Ketoisocaproic acid	Unknown
A	0·16	0·16	0·24	0·24
B	0·70	0·69	0·74,* 0·79	0·74,* 0·78
C	0·53,* 0·66	0·52,* 0·66	0·67, 0·75*	0·67, 0·74*
D	0·48, 0·63*	0·48, 0·62*	0·58,* 0·72	0·57,* 0·73

* Trace. Solvent A, benzene/acetic acid (19:1); B, EtoAc/CH_2Cl_2/88% formic acid (19:19:2); [7]C, $CHCl_3$/acetone/acetic acid (10:10:1); D, 1-butanol/95% ethanol/1% ammonia (6:1:3).

The toluene layer after removal of the acidic DNPH derivatives contained both the neutral DNPHs and the unreacted 2,4-dinitrophenylhydrazine. After evaporation of the solvent, the residues were taken up in chloroform and analyzed by TLC. The sample from the reaction mixture with added leucine gave a spot that appeared to be larger than the control sample from the reaction mixture without added leucine. As shown in Table 2, the R_fs of this spot in four solvents were identical to those of authentic 3-methylbutanal–DNPH, a result confirmed by cochromatography.

TABLE 2. TLC OF 3-METHYLBUTANAL-DNPH PRODUCT RESULTING FROM L-LEUCINE CATABOLISM BY TOMATO ENZYME

	R_f of DNPH	
Solvent	3-Methylbutanal	Unknown
A	0·34	0·34
B	0·16	0·16
C	0·58	0·58
D	0·31, 0·34*	0·31, 0·34*

* Trace. Solvent A, water-saturated benzene;[8] B, benzene/petroleum ether (3:2); C, benzene/EtoAc (20:1);[9] D, benzene/petroleum ether/$CHCl_3$ (2:1:2)

[6] P. SMITH, *J. Chromatogr.* **30**, 273 (1966).
[7] C. MONDER, *J. Biol. Chem.* **242**, 4603 (1967).
[8] L. W. ROONEY, A. SALEM and J. A. JOHNSON, *Cereal Chem.* **44**, 539 (1967).
[9] K. RANDERATH, *Thin-Layer Chromatography* (1st ed.), English translation, p. 217, Academic Press, New York (1965).

The conversion of leucine-^{14}C into these carbonyl compounds was demonstrated by use of L-leucine-U-^{14}C as substrate. The DNPH derivatives of the reaction products were separated as for unlabeled leucine. TLC analyses were carried out for the derivatives, and the chromatograms were then analyzed by radiochromatography. The spot corresponding to α-ketoisocaproic acid gave a sharp peak. The radioactivity of the spots was then determined and the results showed that oxaloacetic and α-ketoglutaric acids were also labeled (Table 3).

TABLE 3. DISTRIBUTION OF ^{14}C-LABELED CARBONYL COMPOUNDS RESULTING FROM L-LEUCINE-U-^{14}C CATABOLISM BY ENZYME PREPARATIONS FROM MATURE GREEN TOMATOES

Compound	Radioactivity (cpm/mg protein/10 min)
α-Ketoisocaproic acid	37,500
α-Ketoglutaric acid	340
Oxaloacetic acid	580
Pyruvic acid	330
3-Methylbutanal	590

The enzyme activity obtained depended on the age of the fruit (Table 4). The preparations from young fruit contained much higher activity than those from mature fruit. A marked decrease in activity was seen as the fruit started to ripen. The enzyme required pyridoxal phosphate for maximum activity. Iodoacetate (10^{-5} M) and *p*-chloromercuribenzoate (6×10^{-6} M) exhibited about 25 and 90 per cent inhibition, respectively. The enzyme activity of the mitochondrial preparations (Table 4) was much lower than that of the supernatant

TABLE 4. PRODUCTION OF α-KETOISOCAPROIC ACID FROM L-LEUCINE BY ENZYME PREPARATIONS FROM TOMATO FRUIT UNDER VARIOUS CONDITIONS

Treatment	α-Ketoisocaproic acid produced (mμmole/mg protein/10 min)
Young fruit, 1 in. dia.	
Mitochondria, complete system*	17
$(NH_4)_2SO_4$ precipitation, complete system	319
Mature pink fruit	
Mitochondria, complete system	0
$(NH_4)_2SO_4$ precipitation, complete system	71
Mature green fruit	
Mitochondria, complete system	16
$(NH_4)_2SO_4$ precipitation, complete system	211
complete system (zero-time)	0
− Pyridoxal phosphate	147
− α-Ketoglutaric acid	15
+ Iodoacetate (10^{-5}M)	158
+ *p*-Chloromercuribenzoate (6×10^{-6}M)	19

* A complete system contained 45 μmoles of Tris-HCl buffer (pH 8·4), 10 μmoles of L-leucine, 10 μmoles of α-ketoglutaric acid, 0·1 μmole of pyridoxal phosphate, and enzyme (0·6 ~ 1·1 mg). Total volume was 1·5 ml. Incubation was conducted at 30° for 10 min. The values are average of two experiments.

fluid regardless of the stage of maturity, and no appreciable activity was found in the mitochondrial preparation from pink tomatoes.

DISCUSSION

The results obtained in this study support earlier observations that leucine could be converted into carbonyl compounds by a tomato extract.[3] It is now evident that these compounds included some keto acids in addition to the 3-methylbutanal reported earlier.[5] It should be noted that in the present study, experiments were conducted in a way different from that reported previously.[3-5] The extraction medium and the method used for the enzyme preparation, the enzyme used in the assay, and the much shorter reaction time employed (10 min vs. 3–5 hr) were some of the new conditions considered to be advantageous.

Under the new experimental conditions, α-ketoisocaproic acid was the predominant reaction product (Table 3). The conversion of the added leucine to this keto acid was approximately 2 per cent within a 10-min incubation period. In tomatoes themselves, the L-leucine: 2-ketoglutarate aminotransferase may be very active.

In contrast to the earlier report by Leonard and Burris on L-aspartate: 2-ketoglutarate aminotransferase,[1] the L-leucine: 2-ketoglutarate aminotransferase in tomatoes was present even when the fruits were very young (Table 4). As some other keto acids such as pyruvate, oxaloacetate, and α-ketoglutarate were also labeled (Table 3), it is obvious that the α-ketoisocaproic acid produced was metabolized further.

Although the data in Table 4 show that α-ketoisocaproic acid production by the enzyme extracts decreases with development of fruit, one cannot conclude that the L-leucine: 2-ketoglutarate aminotransferase activity in the fruits declined accordingly. Not only may enzyme activities *in vivo* be quite different from those *in vitro*, but one must also consider the presence of other enzymes that may affect the conversion of α-ketoisocaproic acid into other compounds. Activities of a variety of enzymes change during fruit maturation and ripening. The amounts of inhibitors or activators may also change during ripening.

The increase in enzyme activity by the addition of pyridoxal phosphate (Table 4) agrees with results obtained with some other aminotransferases.[10,11] Both iodoacetate and *p*-chloromercuribenzoate at the tested concentrations proved inhibitory to the enzyme, indicating that sulfhydryl groups are necessary for the activity, as is true for most aminotransferases.

The L-leucine: 2-ketoglutarate aminotransferase occurred mainly in the soluble portion of the enzyme extract under the experimental conditions used (Table 4). This supports earlier findings with a crude enzyme extract.[3] The activity associated with the mitochondria was less than 10 per cent of that with the soluble fraction, and no enzyme activity was observed in mitochondrial preparations from pink or red tomatoes. It should be mentioned that whereas the mitochondria did not show much L-leucine: 2-ketoglutarate aminotransferase activity, they were highly active in converting L-alanine to pyruvic acid.[12]

EXPERIMENTAL

Materials

The reference 2,4-dinitrophenylhydrazone of 3-methylbutanal was prepared by the method of Shriner *et al.*[13] and recrystallized once from ethanol. Those of the keto acids were prepared by the addition of 0·2%

[10] D. H. BONE and L. FOWDEN, *J. Exptl Botany* **11**, 104 (1960).
[11] R. J. ELLIS and D. D. DAVIES, *Biochem. J.* **78**, 615 (1961).
[12] M. H. YU and M. SPENCER, unpublished results.
[13] R. L. SHRINER, R. C. FUSON and D. Y. CURTIN, *The Systematic Identification of Organic Compounds*, 5th edition, p. 253, John Wiley and Sons, New York (1964).

2,4-dinitrophenylhydrazine in 2 N HCl to their aqueous solutions, and the precipitates collected. No recrystallization was carried out.

Enzyme Preparation

Tomatoes were grown in a greenhouse and fruits were picked and stored at 5° 2–3 hr before use. Unless otherwise stated, mature green tomatoes were used for the preparation of enzyme. Tomatoes (100 g) were cut into pieces and grated in a meat grinder with 100 ml of extraction medium consisting of 0·05 M phosphate buffer (pH 7·6), 0·5 M mannitol, 4 mM EDTA, and 5 mM cysteine. During the grating, the pH of the homogenate was maintained at 7·0 by addition of 1N KOH. The homogenate was pressed through eight layers of cheesecloth, and centrifuged at $3000 \times g$ for 8 min. The supernatant was re-centrifuged at $15{,}000 \times g$ for 25 min to separate the mitochondria and the resulting supernatant used for fractionation by $(NH_4)_2SO_4$. The fraction precipitated between 40 and 55% saturation was suspended in 10 ml of 0·05 M Tris-HCl buffer (pH 8·4), and used immediately. (Dialysis against the same buffer either at pH 8·4 or 7·0 for overnight inactivated the enzyme almost completely). The mitochondria were washed twice with the phosphate buffer and finally taken up in 5 ml of 0·05 M Tris-HCl buffer (pH 8·4), and used immediately. This choice of pH was based on the fact that the enzyme under investigation has rather high optimum pH values.[14,15] Protein was determined by the method of Lowry *et al.*[16]

Assay Conditions

The L-leucine:2-ketoglutarate aminotransferase was assayed by the method of Ichihara and Koyama,[14] with slight modifications. Unless otherwise stated, a complete reaction mixture contained 45 μmoles of Tris-HCl buffer (pH 8·4), 10 μmoles of L-leucine, 10 μmoles of α-ketoglutarate, 0·1 μmole of pyridoxal phosphate, and enzyme. Total volume was 1·5 ml. The reaction mixture was preincubated at 30° for 5 min and the reaction was started by the addition of leucine. Incubation was at 30° for 10 min, and was followed by addition of 0·5 ml of a fresh tungstic acid solution (10% Na_2WO_4/$\frac{2}{3}$N H_2SO_4/H_2O, 1:1:3).[17] The acidified reaction mixture was centrifuged in the cold and the supernatant transferred to a test tube. 2 ml of 0·5% 2,4-dinitrophenylhydrazine in 2 N HCl was added and the mixture was allowed to stand for 30 min at room temperature. The phenylhydrazones formed were extracted into 5 ml of toluene, and the toluene extract was washed with two 5 ml of 0·5 N HCl. 2 ml of the washed toluene layer was mixed with 2 ml of 10% Na_2CO_3, and a 1·5 ml aliquot of the carbonate layer was mixed with 1·5 ml of 1·5 N NaOH. The absorptivity of the pink-colored solution was then determined in a spectrophotometer at 440 nm. A control consisted of all components except the substrate L-leucine and was carried through the same procedure. The difference in absorbance between the control and the sample was considered as the net production of α-ketoisocaproic acid. A standard curve for α-ketoisocaproic acid was constructed with solutions of known concentrations that were carried through the same procedure.

Identification of 2,4-Dinitrophenylhydrazones

The toluene phase containing the phenylhydrazone derivatives was extracted with 10% Na_2CO_3 until the carbonate layer was colorless. The remaining toluene extract was evaporated to dryness and the residue taken up quantitatively in 0·2 ml $CHCl_3$. This solution was then analyzed by TLC. The carbonate extract containing the acidic hydrazones was cooled in an ice bath, acidified with 50% HCl solution, and then extracted with diethyl ether. The ethereal extract was evaporated to dryness, and the residue taken up in 0·2 ml of methanol and then analyzed by TLC on Silica gel G (0·25 mm thick).

Isotopic Studies

The incorporation of L-leucine into carbonyl compounds was studied by adding 1 μc ($2{\cdot}2 \times 10^6$ dpm) of L-leucine-U-^{14}C to each reaction mixture. This gave a radioactivity of about $1{\cdot}8 \times 10^6$ cpm under the defined conditions. Other conditions were the same as with unlabeled amino acid, except that the washing of the toluene extract with 0·5 N HCl was omitted. After development, the TLC plates were heated in an oven to remove the solvent, and the chromatograms analyzed by radiochromatography using a Nuclear-Chicago Actigraph III with a model 1002 TLC attachment (Nuclear-Chicago Corp.). For determination of the radioactivity, the spots were scraped off the plate and placed into scintillation vials containing 0·5 g of Cab-o-sil,

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[15] R. T. Taylor and W. T. Jenkins, *J. Biol. Chem.* **241**, 4396 (1966).
[16] D. H. Lowry, N. J. Rosebrough, A. L. Farr and R. L. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[17] W. J. P. Neish, in *Methods of Biochemical Analysis* (edited by D. Glick), Vol. 5, p. 107, Interscience Publishers, N.Y. (1957).

15 ml of scintillation solution[18] was added to the vial, and the countings were performed in a Nuclear-Chicago scintillation spectrophotometer Unilux II.

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