Phytochemistry, 1968, Vol. 7, pp. 561 to 565. Pergamon Press Printed in England

PRECURSORS OF VOLATILE COMPONENTS IN TOMATO FRUIT—III.

ENZYMATIC REACTION PRODUCTS

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(*Received* 14 *April* 1967)

Abstract—The volatile components produced by enzyme extracts from tomato fruit using amino acid substrates were analysed by gas and thin-layer chromatography. Alanine, leucine, and valine were among the best substrates. Extracts from red-ripe tomatoes were more active than those from green fruit. The products differed somewhat with each preparation and with the particular substrate used. Propanal was present in most reaction products especially when alanine was used as substrate. With enzymes from red-ripe fruit, alanine gave mainly carbonyl compounds while leucine and valine gave mainly alcohols. Mixed amino acids sometimes resulted in new volatile components. The mechanism appears to involve transamination.

INTRODUCTION

PREVIOUS work based on spectrophotometric evidence has shown that enzymes from tomato fruit were capable of converting alanine, aspartic acid, leucine and in some cases valine to carbonyl compounds.[1] The technique of gas chromatography was used by Weurman[2] to investigate volatile enzyme products from raspberries and the same method has been applied to the present study. The production of 3-methylbutanal and 3-methyl-1-butanol from L-leucine by enzymes from greenhouse-grown red-ripe tomatoes has been reported previously.[3] Recent work with labeled [^{14}C]-L-leucine has confirmed the conversion of this amino acid to 3-methylbutanal.[4] The present paper deals with the enzyme reaction products in considerable detail, using enzymes from field-grown tomatoes, and the results support earlier findings[1] that amino acids can serve as precursors in the production of volatile components in tomato fruit.

RESULTS

Gas Chromatography

The products extracted by ether from the reaction mixtures varied with the identity of the amino acid substrate, the incubation period, and with the particular enzyme preparation. This last appeared to be the most important factor affecting the chromatographic pattern as no appreciable differences were observed with different amino acids if enzyme preparations were omitted from the reaction mixtures. The control, which contained an enzyme preparation and all the components except substrate amino acids, occasionally gave one or two very

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[1] M. H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **7**, 555 (1968).
[2] C. WEURMAN, *Food Technol.* **15**, 531 (1961).
[3] M. H. YU, D. K. SALUNKHE and L. E. OLSON, presented at the 152nd Meeting, ACS, New York, N. Y., Sept. (1966).
[4] M. H. YU, D. K. SALUNKHE and L. E. OLSON, manuscript in preparation.

small peaks, probably from the enzyme preparation itself. In general, differences in the chromatograms can, therefore, be considered to be due to enzymatic reactions.

Figure 1 shows the chromatograms obtained from the ether extracts of reaction mixtures which contained an enzyme preparation from red-ripe tomatoes. Using a carbowax 20 M column, five distinct peaks were obtained when both leucine and alanine were used as substrates. Valine gave four peaks, whereas using aspartic acid only two small peaks were

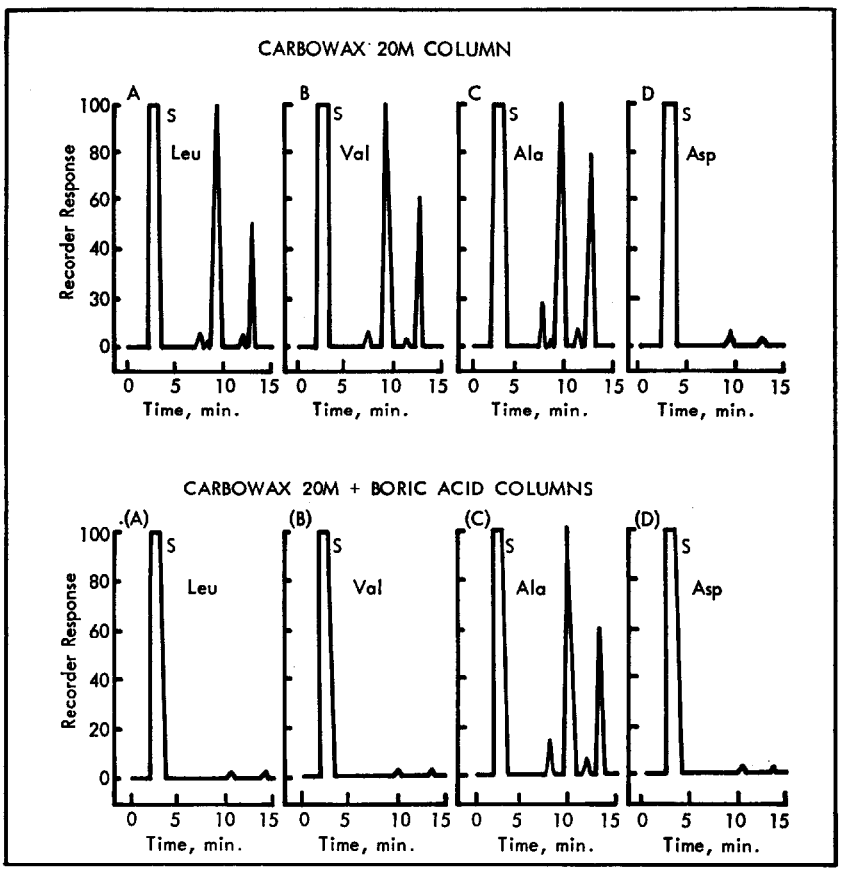

FIG. 1. PRODUCTION OF VOLATILE COMPONENTS FROM AMINO ACIDS BY CRUDE PREPARATIONS FROM FIELD-GROWN TOMATOES AT THE RED-RIPE STAGE.

A to D are chromatograms obtained by using carbowax 20 M column, whereas (A) to (D) using carbowax 20 M and boric acid columns. The amino acid shown in each chromatogram is that used as substrate(s). The first peak in each chromatogram labelled "S" is the ether used as solvent.

obtained. On passing the ether extracts through both carbowax 20 M and boric acid columns,[5] most of the compounds produced by those amino acids except alanine were removed, indicating that they were probably alcohols (Fig. 1(A) to (D)). Surprisingly, the chromatograms obtained from the reaction mixture which contained alanine as substrate did not change appreciably after boric acid treatment. This indicates that the peaks represent carbonyl compounds, tertiary alcohols, or esters. One of the peaks (Chromatogram C, No.

[5] R. M. IKEDA, D. E. SIMMONS and J. D. GROSSMAN, *Anal. Chem.* **36**, 2188 (1964).

3 peak) which remained after treatment with boric acid had the same retention time as that of authentic propanal. This was confirmed by the enrichment technique and by TLC of its 2,4-dinitrophenylhydrazone. Efforts to identify the compounds represented by other peaks were not successful.

The residue and the washings from the ether extract of the enzymatic reaction mixtures were analyzed by TLC for changes in the amino acids used as substrates. It was found that the size of the spot of each substrate amino acid was smaller than the control. In addition, each of the reaction mixtures containing the enzyme preparation showed enlargement of a spot which was subsequently identified as glutamic acid. The most marked changes were observed when aspartic acid was the substrate. The glutamic acid content of the reaction mixtures was determined manometrically by using glutamic acid decarboxylase, and the results confirmed that glutamic acid was produced in the enzymatic reaction. Table 1 shows the data obtained from an experiment involving enzymes from tomatoes at the large green stage.

TABLE 1. ENZYMATIC PRODUCTION OF GLUTAMIC ACID BY TOMATO EXTRACTS FROM FIELD-GROWN TOMATOES AT THE LARGE GREEN STAGE

	Substrate†			
	Alanine	Aspartic acid	Leucine	Valine
Glutamic acid formed (μ mole)	8·7*	10·6	6·9	9·6
Amino acid converted (%)	17·4	21·2	13·4	19·2

* Correction has been made for the glutamic acid originally present in the enzyme extracts. Glutamic acid was determined manometrically using L-glutamic acid decarboxylase.

† 50 μ moles of each.

The presence of ethanal in tomatoes has been reported by several workers.[6–8] It was possible that this aldehyde might be formed during the catabolism of alanine. Qualitative analyses were made to detect ethanal during the incubation period as well as at the end of the enzymatic reaction. The reaction mixtures containing alanine and the control which contained no added amino acid were analyzed, according to the methods of Feigl.[9] The results failed to confirm the presence of ethanal. When an authentic sample of ethanal was used, the reagent immediately gave the characteristic color upon contact with the vapor.

DISCUSSION

The production of 3-methylbutanal and 3-methyl-1-butanol from L-leucine by enzymes from greenhouse-grown tomatoes has been reported previously.[3] These two compounds were not detected by GLC in the present study. Whether this is due merely to different enzyme preparations is not known.

The enzymatic reaction products differed not only with enzyme source, but also with substrates, incubation period, and perhaps extraction methods. The first appears to be the

[6] M. S. SPENCER and W. L. STANLEY, *J. Agric. Food Chem.* **2**, 1113 (1954).
[7] F. F. MATTHEWS, *Dissertation Abstr.* **21**, 1693 (1961).
[8] Y. V. RAKITIN, *Biokhimiya* **10**, 373 (1945).
[9] F. FEIGL, *Spot Tests in Organic Analysis* (5th ed.), p. 334. Elsevier, New York, N. Y. (1956).

most important factor. Enzyme preparations from fruit of different locations (e.g. fruit grown in greenhouses or in field), varieties, maturity stages, all seem to have an effect. For example, more enzymic activities appear to be present in the field-grown tomatoes than the greenhouse-grown fruit, since the products from the former preparations contained more volatile components (Fig. 1A and ref. 3). As shown in Fig. 1, a large amount of propanal was produced from alanine by enzymes from field-grown red-ripe tomatoes. Surprisingly, this aldehyde was also produced from leucine by an enzyme preparation from greenhouse-grown young tomatoes of unidentified variety.[10] The production of this aldehyde from 1-propanol has recently been reported by Meigh *et al.*[11] With the enzyme extracts used in the present study, no appreciable amount of carbonyl compounds or esters could be detected in the reaction products when valine was the only substrate (Fig. 1). Using a preparation from commercial tomato fruit, however, valine gave a distinct peak which remained after the boric acid treatment.[12]

The results obtained with aspartic acid were exceptional. While in many experiments this amino acid resulted in an increased absorbance compared with the control,[1] the ether extract of the enzymatic reaction mixtures did not give significant changes in chromatographic patterns. Since the present study is limited to large green and red-ripe tomatoes, it is possible that aspartic acid may be utilized as a precursor at other stages of ripeness. It is also possible that the reaction products of this amino acid may be more soluble in water than in ether, and thus were not detected in the chromatograms.

The anzymatic conversion of amino acids to volatile components appears to involve transamination (Table 1). It should be noted that those amino acids that could be precursors of volatile components are those which are comparatively abundant as free amino acids, or those whose concentrations changed markedly during ripening of the fruit.[13] The work reported by other workers concerning the changes in pyruvate and α-ketoglutarate in growing tomatoes is of interest. According to Andreotti and Barbieri,[14] α-ketoglutaric acid increased from 8·4 to 22·7 mg, and pyruvic acid decreased from 65 to 6·2 mg per 100 g dry matter during ripening. In ripening tomatoes, the increased amount of α-ketoglutaric acid could be important for the transamination to proceed smoothly. On the other hand, the utilization of pyruvic acid in the synthesis of volatile compounds such as propanal noted in this study, may partly account for the decrease in the concentration.

Although the experiments conducted in this investigation were originally based upon the formation of carbonyl compounds and subsequent studies limited to a few amino acids, it must be emphasized that other amino acids and related compounds should not be excluded as possible precursors. Those amino acids that did not result in an increase in carbonyl compounds may give rise to other volatile components such as alcohols. An example is valine and in many experiments this amino acid did not give increased absorbance readings, yet results of gas chromatography showed the production of some alcohols (Fig. 1).

EXPERIMENTAL

The methods employed for enzyme preparation were the same as described previously.[1] The assay conditions were also the same except that the amounts of the reaction mixtures were increased 10- to 20-fold, and the reaction was allowed to proceed in 125 ml Erlenmeyer flasks. After incubation, the reaction mixtures

[10] M. H. YU, Ph. D. Thesis, Utah State University, Logan, Utah (1967).
[11] D. F. MEIGH, H. K. PRATT and C. COLE, *Nature* **211**, 419 (1966).
[12] M. H. YU, L. E. OLSON and K. D. SALUNKHE, unpublished data.
[13] M. H. YU, L. E. OLSON and D. K. SALUNKHE, *Phytochem.* **6**, 1457 (1967).
[14] R. ANDREOTTI and G. BARBIERI, *Ind. Conserve (Parma)* **34**, 7 (1959).

were extracted with ether, and the condensed ether extract was subsequently analyzed by gas chromatography,[3] using a MicroTek gas chromatograph, GC2500R (MicroTek Inst., Inc., Baton Rouge, La.). The columns were made up of two 3 m × 6 mm stainless steel tubes packed with chromosorb DMS (60–80 mesh) which was impregnated with 10 per cent carbowax 20 M. A flame ionization detector was employed, and the temperature programmed from 75 to 220° at a rate of 4 deg per min.

The identification of propanal was performed by using gas chromatography and TLC. Gas chromatographic identification consisted of comparison between the retention time of the sample and authentic propanal, removal of alcohols from the sample,[5] and then the enrichment technique. In TLC a portion of the ether extract used in the gas chromatography was utilized for preparing the 2,4-dinitrophenylhydrazone by addition of 0·2 per cent 2,4-DNP in 2N HCl. The mixture was evaporated to dryness and the residue was taken up in a small amount of $CHCl_3$ and was used as a sample. Chromatograms were developed on silica gel G plates (0·25 mm thick) in benzene/hexane/chloroform in the proportion of 2:2:3 by volume.[10] The R_f value was compared with that of the DNP derivative of authentic propanal.

Changes in the substrate amino acids during the reaction were followed by TLC. The procedures were based upon those described by Randerath.[15] Silica gel G was used as coating material (0·25 mm thick). The developing solvent system consisted of 1-butanol/acetic acid/water (60:20:20, by volume). After development the plates were dried at 110° for 10 min, sprayed with 5 per cent ninhydrin in acetone and heated at 110° for 10 min.

Quantitative determination of glutamic acid was conducted manometrically by using L-glutamic acid decarboxylase (purchased from Nutritional Biochemical Co., Cleveland, Ohio). The methods of Gunsalus and Stamer,[16] and also of Cohen[17] were employed. Samples used for the determination were prepared as follows. The residues from the ether extraction and the washings were combined and the mixture was freed from the ether by evaporation on a water-bath. The mixture was then centrifuged and the supernatant was evaporated under reduced pressure in a Rotavapor to 2–3 ml. This residue was made up to 10 ml with distilled water after adjusting the pH to 5·0. A 1 ml aliquot was used for the glutamic acid determination.

Acknowledgements—This investigation was supported by U.S. Public Health Service Research Grant UI 00449 from the National Center for Urban and Industrial Health.

[15] K. Randerath, *Thin-Layer Chromatography* (2nd ed.), p. 110, Academic Press, New York, N. Y. (1966).
[16] I. C. Gunsalus and J. R. Stamer, *Meth. Enzymol.* Vol. II, p. 174 (1955).
[17] P. P. Cohen, *Meth. Enzymol.* Vol. II, p. 178 (1955).