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PRECURSORS OF VOLATILE COMPONENTS IN TOMATO FRUIT—II.

ENZYMATIC PRODUCTION OF CARBONYL COMPOUNDS

MING-HO YU,* L. E. OLSON and D. K. SALUNKHE

Department of Plant Science, Utah State University, Logan, Utah, U.S.A.

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Abstract—Crude enzyme preparations from fresh tomatoes in the presence of certain amino acids are capable of producing carbonyl compounds. The activities of the enzyme extracts from field-grown tomatoes were generally higher than those grown in greenhouses. Enzyme activities increased with the maturity of the fruit, whereas the protein content of the extracts tended to decrease. Three amino acids used as substrates gave distinctive responses with the enzyme and the activity in general appears to be associated with the insoluble part of the preparations.

INTRODUCTION

ALTHOUGH studies on the isolation and identification of aroma components in tomato fruit are numerous,[1–5] those concerned with their biosynthesis are rather limited. Rakitin[6] reported that the greatest amounts of ethanol and acetaldehyde were always found during ripening. Dalal *et al.*[7] showed that many volatile components increased progressively during the development of the fruit. A marked decrease in the concentrations of several amino acids during ripening has been shown previously.[8–9] It was considered[9] that some relationship might exist between the diminution of the amino acids and the production of the volatile components in tomatoes. To verify this hypothesis an enzymatic study seemed appropriate. This paper reports evidence obtained from *in vitro* studies indicating that fresh tomato extracts can convert certain amino acids to carbonyl compounds.

RESULTS

The enzyme preparations contained 3–5 mg of soluble protein per ml extract, depending on the maturity of the fruit from which enzymes were extracted. The protein content of the preparations from green tomatoes was generally higher than that from ripe fruit. The highest concentration was usually found at the fourth stage, a marked decrease occurring between the fifth and the sixth stages of maturity (Table 1).

* Present address: Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada.

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TABLE 1. SOLUBLE PROTEIN OF ENZYME EXTRACTS AT VARIOUS STAGES OF MATURITY

Enzyme source	Stage of maturity								
	1	2	3	4	5	6	7	8	9
	Protein concentration (mg/100 g fresh tomato)*								
Greenhouse-grown tomatoes	—	—	—	634	616	475	401	446	468
Field-grown tomatoes	627	603	612	665	565	450	467	492	532

* Values are an average of two determinations.

The enzyme preparation was found to be capable of converting leucine to certain carbonyl compounds which were determined by their reaction with dinitrophenyl hydrazine (DNP). As shown in Table 2, without enzymes, the absorbance of DNP-reacting compounds produced from a mixture containing α-ketoglutaric acid and pyridoxal phosphate was higher than that from a mixture to which leucine was added. With the enzyme preparation, the latter mixture resulted in higher absorbance. Figure 1 shows the effect of incubation period on the enzymatic activities of two preparations. Although different preparations showed varying rates of activity, an increased incubation period generally gave higher absorbance readings. Under the experimental conditions, a period of 4 to 6 hours usually produced the highest activity. The enzymatic reaction appears to belong to zero order up to the first hour of incubation.

TABLE 2. ABSORBANCE OF VARIOUS REAGENTS WITH AND WITHOUT ENZYME PREPARATIONS*

Composition	Absorbance	
	No enzyme	With enzyme
Buffer solution only	0	0·159
Leucine	0·002	0·160
α-Ketoglutaric acid	0·068	0·219
Pyridoxal phosphate	0·123	0·246
Leucine + α-ketoglutaric acid	0·073	0·224
Leucine + Pyridoxal phosphate	0·128	0·247
α-Ketoglutaric acid + Pyridoxal phosphate	0·185	0·284
Leucine + α-Ketoglutaric acid + Pyridoxal phosphate	0·172	0·296

* Enzymes were prepared from greenhouse-grown tomatoes of the large green stage. Each reaction mixture contained 200 μ moles of Tris-HCl buffer, pH 8·4, and 1·0 ml of crude enzyme extract (3·47 mg of protein), and where indicated in the table: 2·5 μ moles of leucine, 1·14 μ moles of α-ketoglutaric acid, and 0·07 μ moles of pyridoxal phosphate. Total volume was 2·0 ml. Incubation was carried out for 3 hr at 37°. At the end of experiment, 1 ml of 0·0125 per cent 2,4-dinitrophenylhydrazine in 2 N HCl was added, and the mixture was incubated for 10 min more. Two ml of H_2O were then added followed by 5 ml of 0·6 N NaOH. After centrifugation, the absorbance of the supernatant was read at 510 nm.

When the enzyme preparation was boiled, or was extracted from tomatoes previously frozen for one month or so at −20°, the absorbance of both sample and control did not differ appreciably. When freshly prepared enzymes were kept for some time, even in the cold, the activity also diminished gradually.

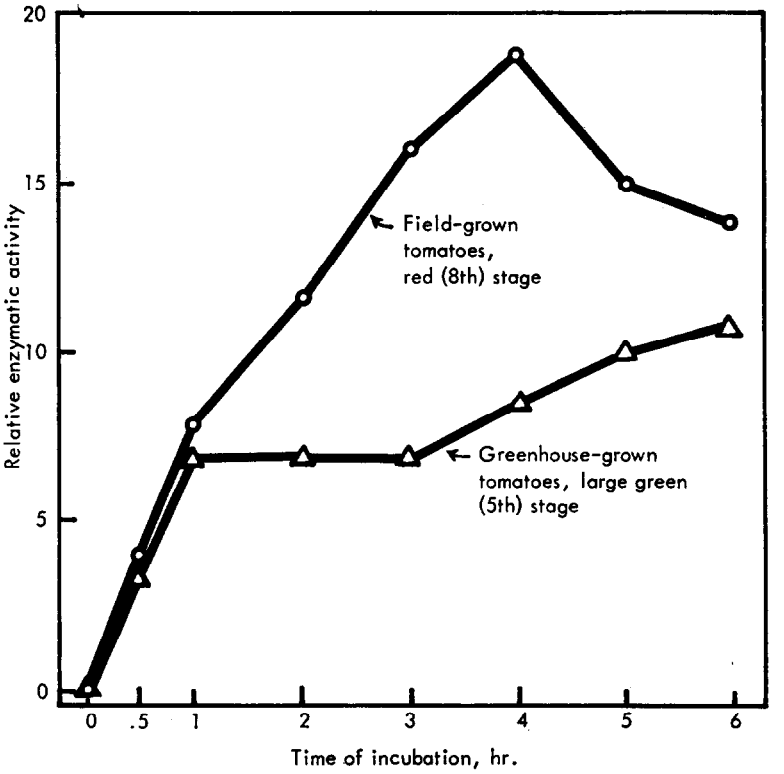

FIG. 1. EFFECT OF INCUBATION TIME ON ENZYMATIC ACTIVITY OF TOMATO EXTRACTS IN CONVERTING LEUCINE TO CARBONYL COMPOUNDS.

Other amino acids were then tested as suitable substrates; enzyme preparations from tomatoes at various stages of maturity were used for this purpose. As with leucine, alanine and aspartic acid almost always resulted in increased DNP-absorbance, varying with the maturity of the fruit. Other amino acids showed either a decrease from the control or did not respond at all. Valine and isoleucine, although structurally similar to leucine, did not behave similarly and in many instances gave a decreased absorbance compared with the control.

Enzyme preparations from tomatoes at different maturity stages showed varying activities in converting amino acids to carbonyl compounds. As shown in Table 3, the enzymes from young fruit had low activities; the activities increased gradually with the maturation of fruit. This trend is true especially for alanine and leucine. For these two amino acids, the activities of preparations from the field-grown tomatoes peaked at the 7th or pink stage of maturity, and then declined. By contrast, no clear-cut pattern in the change in activities with fruit maturation was observed for aspartic acid. Table 3 also shows the comparison of enzymatic activities between the greenhouse-grown and the field-grown tomatoes; the activities were generally higher in the preparations from field-grown fruit.

A marked difference in the enzymatic activity was observed between the soluble and the insoluble fractions of the extracts (Fig. 2). The activities of the soluble fractions did not change appreciably with rate of centrifugation or with substrate amino acids. Those of the insoluble fractions, however, differed with both these factors. For leucine, the activity of the insoluble fraction increased somewhat when centrifugation was conducted at 9,000 g, but no

TABLE 3. COMPARISON OF ENZYMATIC ACTIVITIES BETWEEN PREPARATIONS FROM FIELD-GROWN AND GREENHOUSE-GROWN TOMATOES

Substrate		Relative enzymatic activity: Stage of maturity 4	5	6	7	8	9
Alanine	F*	9	25	27	34	19	12
	G†	8	—‡	—	10	13	25
Aspartic acid	F	5	13	4	9	3	1
	G	0	—	—	0	0	0
Leucine	F	9	11	16	23	19	18
	G	4	8	3	6	11	13

* Enzymes prepared from field-grown tomatoes.
† Enzymes prepared from greenhouse-grown tomatoes.
‡ No determination was carried out.

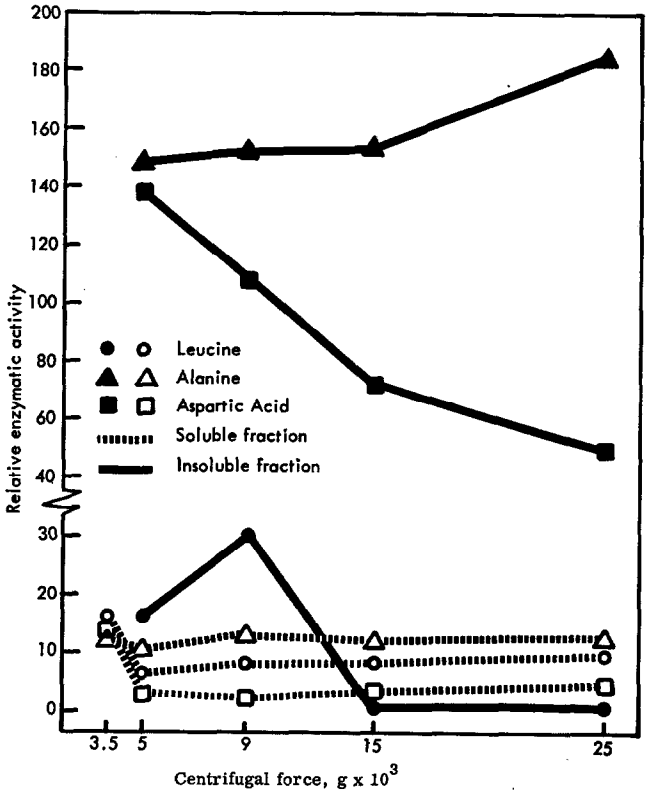

FIG. 2. COMPARISON OF ENZYMATIC ACTIVITIES BETWEEN SOLUBLE AND INSOLUBLE FRACTIONS OF ENZYME EXTRACTS OBTAINED BY DIFFERENTIAL CENTRIFUGATION.*

* Crude enzymes were extracted from field-grown tomatoes at the pink (7th) stage. Centrifugation was conducted at the stated rate for 10 min.

activity was observed with preparations obtained beyond this rate. The insoluble fraction had strikingly high activities for the conversion of both alanine and aspartic acid. The increase in activity was about 15 to 20-fold for alanine, and 10 to 60-fold for aspartic acid, respectively, compared with the soluble fractions.

DISCUSSION

Results obtained from the present study have indicated that amino acids could serve as the precursors for the production of certain carbonyl compounds. This supports the suggestions of other workers in their studies on different materials.[10–11] Among the amino acids that were tested, alanine, aspartic acid, and leucine appeared to be important substrates. It is interesting to note that these amino acids are present in comparatively high concentrations in the fruit and they decreased markedly during ripening.[9] It appears that at least part of the amino acids originally present in the free state could be utilized in the production of carbonyl compounds.

The enzyme extracts from young green tomatoes usually contained more protein (Table 1), but their activity was lower than those from mature fruit (Table 3). This indicates that further enzymes responsible for the conversion of amino acids to carbonyl compounds are synthesized with the climacteric. After peaking at the pink stage of maturity, the enzymes from field-grown fruit decreased in activity in converting both alanine and leucine (Table 3). This may be due to the possible increase in inhibitors during the ripening period. It is also possible that the carbonyl compounds that have been produced may partly be utilized for the synthesis of other compounds, such as acids, alcohols, or even esters. This consideration is justified to some extent by the findings of Dalal,[12] according to which the production of carbonyl compounds started as early as in the green fruit and the concentrations of both alcohols and esters were higher in ripe tomatoes than in immature fruit.

The results shown in Fig. 1 could be explained in a similar manner. When enzymes from large green tomatoes were used, the activity usually increased with time of incubation. When enzyme preparations from ripe fruit were used, however, the activity almost always increased up to 4 or 5 hr of incubation and then declined. While enzyme inhibition or volatilization of the products could account for the latter phenomenon, it is also possible that the synthesized carbonyl compounds may be converted to either alcohols or acids.

The presence of a marked difference in enzymatic activities between the soluble and the insoluble fractions of the extracts is noteworthy (Fig. 2). This may indicate that the sites of enzymes responsible for conversion of each substrate amino acid are different. The enzymes concerned with the conversion of leucine appear to be located in the soluble fraction, whereas those concerned with alanine and aspartic acid are in the mitochondria. This is in a way consistent with the findings of Wilson *et al.*,[13] and Bone and Fowden,[14] that the alanine–glutamic acid and the aspartic acid–glutamic acid transaminases are located primarily in the mitochondria of plant cells. Transamination may be involved in the production of carbonyl compounds from those amino acids used in the present study. This process may account for the presence of a high concentration of glutamic acid in tomato fruit.[8–9]

Although the results of this study strongly suggest the formation of carbonyl compounds

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from some amino acids in the presence of crude enzyme extracts, the nature of the carbonyl compounds is not known. Some of the compounds may be volatile whereas others may be not. Part of this problem was solved by analysis of the enzymatic reaction products using gas chromatography, details of which are reported in Part III of this series.

EXPERIMENTAL

Materials

Both greenhouse- and field-grown tomatoes, V. R. Moscow variety, were employed in this work. The methods used in classifying and sampling the fruit were as described previously.[9]

Preparation of Enzyme Extract

100 g of fresh tomatoes were blended in a Waring blendor containing an equal amount (w/v) of 0·1 M Tris-HCl buffer, pH 8·4, for 1 min. The slurry was ground in a cold mortar with sand for 2–3 min. The mixture was then strained through 4 layers of cheesecloth and centrifuged at 3500 *g* for 8 min. The supernatant was used as crude extract in most experiments. To compare the enzymatic activities of the soluble and insoluble fractions (Fig. 2), the crude extracts were divided into 5 portions: one portion was used as such (3500 g), while the others were centrifuged for 10 min at 5000, 9000, 15,000, and 25,000 *g* respectively. The supernatant from each centrifugation was separated and used as such, whereas the precipitates were each washed with 10 ml of the same buffer, recentrifuged for 10 min at the same rate at which they were originally precipitated. The supernatant was discarded, and the pellets were mixed with 10 ml of the buffer and used as the insoluble fraction.

Protein Determination

The protein content of the enzyme extracts was determined by the method of WADDELL.[15] The extracts were usually diluted 100 times with 0·9 per cent NaCl and the absorbance of the diluted mixture was read in a Beckman DU spectrophotometer at 215 and 225 nm. The saline diluent solution was used as blank.

Assay Conditions

Enzyme assays were carried out in a test tube containing 1 ml of substrate solution and 1 ml of the enzyme extract. The substrate solution was made up of 0·1 M Tris-HCl buffer, pH 8·4, which contained (per ml) 2·5 μ moles of the amino acid to be tested, 1·14 μ moles of α-ketoglutaric acid, and 0·07 μ moles of pyridoxal phosphate.[16] The reaction was initiated by adding the enzyme extract to the substrate solution. The mixture was incubated at 37° for 5 hr with an occasional agitation, and then assayed for the production of carbonyl compounds by the method of Kachmar and Boyer.[17] A blank consisted of 2 ml of the buffer solution carried through the same procedure. Since the substrate solution and the enzyme extract both contained DNP-reacting substances which absorb at the same wavelength under the experimental conditions, a zero time treatment was employed as control for each sample mixture to minimize errors. For this purpose, the substrate solution and the enzyme extract were incubated separately and, at the end of incubation, the enzyme extract was pipetted in the substrate solution followed immediately by addition of 2,4-dinitrophenylhydrazine. The other procedures were as those employed for the sample. The absorbance of the zero time treatment was subtracted from that of the corresponding sample, and the difference was considered as the net increase due to enzymatic reaction. For each treatment, duplicate samples were used and the values were averaged.

Relative Enzymatic Activity

One unit of relative enzymatic activity was defined as the net increase in absorbance by a 0·001 unit in the Beckman DU Spectrophotometer using a 1·0 cm cell per mg protein per unit incubation period.

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