The Enzymatic Regeneration of Volatile Flavor Components in Carrots

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Experiments were conducted which demonstrated a characteristic raw carrot aroma could be regenerated from essentially odorless carrot substrates ("precursor" material) when the substrates were reacted with "flavorese" (flavor forming) enzymes prepared from raw carrots. "Flavorese" enzyme activity was erratic, raw carrot aroma not always being regenerated with enzyme treatment. A gas-entrainment, on-column trapping technique in conjunction with gas-liquid chromatography-mass spectrometry (glc-ms) was used for examining the headspace volatiles present in enzyme reaction mixtures. This technique provided no reproducible evidence for the enzymatic formation of volatile compounds coinciding with the enzymatic regeneration of raw carrot aroma. The compound(s) responsible for carrot aroma may be potent and present in very low concentrations beyond the limits of detection of the method used, or very labile and unstable under the analysis conditions.

For several years food scientists have been exploring the possibility of improving the flavor of processed food products by treatment with enzymes capable of regenerating volatile flavor compounds lost during processing. The role of enzymes in the regeneration of fresh flavor in processed foods is embodied in the "flavorese" concept of Hewitt *et al.* (1956). This theory advanced the concept that whereas heat processing destroys or inactivates "flavorese" enzymes (enzymes that synthesize flavor compounds), the precursors are sufficiently nonvolatile and sufficiently stable to survive processing. On addition of the appropriate "flavorese" enzyme prepared from the fresh product, nonvolatile precursor(s) are converted to volatile flavor components. This concept has been confirmed by several workers (Schwimmer, 1963) and has been reviewed by Hewitt (1963), Konigsbacher and Hewitt (1964), and Reed (1966). The nature of the substrate ("precursor"), flavor, and enzymes has been fairly well established for a few vegetables which have strong characteristic aromas (cabbage, mustard, horseradish, onion; Reed, 1966; Schwimmer, 1968). Recently, Yu *et al.* (1968a,b) have shown amino acids can be converted into carbonyl compounds and alcohols by crude enzyme extracts prepared from fresh tomatoes. However, no correlations were made between compounds enzymatically formed and the characteristic fresh tomato aroma. Similarly, Myers *et al.* (1970) have reported that the incubation of ripe banana tissue discs resulted in biological conversion of L-leucine to isoamyl alcohol and isoamyl acetate, which are major banana aroma components. Konigsbacher and Hewitt (1964) claim the enzymatic enhancement of processed green bean aroma may be due to a small increase in the concentration of 2-*trans*-hexenal. Similarly, Attaway and Metcalf (1966) indicate a slight enzymatic improvement in orange juice aroma may be due to a small increase in the concentration of limonene. In both of these instances the "enzymes" were not reported to be producing new compounds, but were increasing the concentration of existing compounds.

Owing to the elusive nature of the compound(s) responsible for raw carrot aroma (Buttery *et al.*, 1968; Heatherbell *et al.*, 1970) an investigation using the "flavorese" concept appeared an alternative approach for determining what volatile(s) was important in carrot flavor. Preliminary experiments using crude enzyme preparations and substrates prepared from carrots confirmed this concept, and further investigation of the volatiles being released enzymatically offered an attractive system for elucidating which compound(s) was responsible for carrot aroma and for studying the biosynthesis of a flavor.

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EXPERIMENTAL

Preparation of "Flavorese" Enzymes. All operations were carried out in a 1° C room. Several methods of preparing active "flavorese" extracts were attempted. (1) Acetone powder (A) was prepared by a method similar to that used by Schwimmer (1963). With equipment precooled at −20° C, 100 g of diced carrots were homogenized for 1 min in 100 ml chilled distilled water in a Waring Blendor. The homogenate was squeezed through four layers of cheesecloth and the filtrate centrifuged at 5000 × *g* 10 min. The protein fraction was precipitated from the resulting supernatant by the addition of acetone at −30° C. Acetone was added in the proportions of 5 volumes of acetone/1 volume of supernatant, with gentle stirring over a 10 min period. The suspension was filtered by suction in a Buchner funnel using Van Waters and Rogers White Crepe No. 617 paper to facilitate rapid filtration. The residue was washed several times in the funnel with 200 ml portions of cold acetone. The resulting powder was dried for a few minutes in air to remove excess solvent, and then left for 16 hr at 1° C under vacuum over P_2O_5 to remove remaining traces of moisture and solvent. The dried powder was stored at −30° C under nitrogen in tightly capped jars. Yield ranged from 0.2–0.5% on a fresh weight basis.

(2) Acetone powder (B) was prepared in the presence of polyvinylpyrrolidone (PVP) using a modification of the procedure described by Loomis and Battaile (1966). Diced carrots (200 g) were frozen in liquid nitrogen and blended for 30 sec in a Waring Blendor. The liquid nitrogen frozen powder was suspended in 600 ml of 0.1 *M* phosphate buffer, pH 7.4, containing 200 g of PVP. This suspension was gently stirred for 30 min, then squeezed through a nylon cloth. The residue was reextracted with 400 ml of buffer and the combined filtrates centrifuged at 20,000 × *g* 15 min to remove particulate matter. From the clear supernatant the proteins were precipitated, filtered off, washed, dried, and stored in the same manner as described for powder (A). The yield was 0.2% on a fresh weight basis.

(3) Acetone powders were also prepared by direct extraction of carrot tissue with acetone and acetone containing polyethylene glycol (PEG) by following the method described by Arakji and Yang (1969). These powders labeled (C) and (D),

respectively, were recovered in yields of approximately 6% on a fresh weight basis.

Enzyme extracts for examining "flavorese" activity were prepared from the powders by dissolving (suspending) 50–200 mg of powders (A) or (B) in 25 ml of distilled water of 0.1 *M* phosphate buffer, pH 6.5, for 15 min at room temperature. Extracts from powders (C) and (D) were prepared by suspending 2–6 g of powder in 125 ml of water or buffer, and stirring for 3 hr at 1° C before filtering through a glass wool plug.

Preparation of Substrate ("Precursor") Material. The following sources of "precursor" material were investigated. (1) Freeze dried "precursor"—Diced carrots ($^1/_4$ in.) were blanched to negative peroxidase activity (3 min at 210° F) and homogenized in a Waring Blendor in the proportions of 2 lb carrots/1 l. of water. The homogenate was steam distilled at a temperature of 20–22° C and a pressure of 0.5 mm Hg for 3 hr in one case and for 8 hr in a second preparation. The deodorized homogenate was freeze dried, powdered, and stored under nitrogen at −30° C in tightly capped jars. On reconstitution, this product, which represented 10% of the fresh weight, was bland to the taste, essentially odorless, and not recognizable as carrot.

(2) Nitrogen purged "precursor"—250 g of diced carrots were blanched in 600 ml of boiling distilled water for 2 min, cooled in an ice bath to 30° C, homogenized in a Waring Blendor for 60 sec, and filtered through four layers of cheesecloth. The filtrate was gently stirred in a beaker on a combination hot plate-magnetic stirrer at a temperature of 40° C for periods of up to 3 hr. Removal of volatiles was aided by entrainment in a stream of nitrogen which was bubbled through the extract at a rate of 400 ml/min. The final product, which was used for analysis immediately, was essentially odorless and not recognizable as carrot. (3) Nitrogen purged cooked "precursor"—250 g of diced carrots were boiled in 600 ml of water for 30 min and homogenized, and nitrogen purged as previously described. The final product retained a cooked carrot aroma.

Determination of Flavorese Activity: Flavor Regeneration. The conditions used were similar to those reported by earlier workers (Schwimmer, 1963; Hewitt, 1963). For each test the following samples were prepared: an enzyme and "precursor" reaction sample; a "precursor" only control sample; an enzyme only control sample; and a "precursor" and heat inactivated control sample. These samples were incubated in 250 ml glass stoppered conical flasks in a shaking water bath at 37° C for the desired time (usually 90 min) before being analyzed. Detailed conditions used for each sample were as follows

(1) Enzyme and "precursor" sample—In each instance "precursor" and enzyme were mixed in a final volume of 125 ml of distilled water or 0.1 *M* phosphate buffer, pH 6.5. Combinations examined included (a) 25 ml enzyme extracts of acetone powders (A) or (B) added to 4 g of freeze dried "precursor" which had been reconstituted to 100 ml at 37° C for 15 min; (b) 50–200 mg of acetone powders (A) or (B) directly suspended in 125 ml of nitrogen purged "precursor," and (c) 4 g of freeze dried "precursor" directly reconstituted in 125 ml enzyme extracts from acetone powders (C) or (D).

(2) "Precursor" only control sample—This control consisted of 4 g of freeze dried "precursor" reconstituted to a final volume of 125 ml in water or buffer; or 125 ml of nitrogen purged "precursor" material.

(3) Enzyme only control sample—Enzyme extracts were made up to a final volume of 125 ml in water or buffer.

(4) "Precursor" and heat inactivated control sample—"Precursor" material, and enzyme extracts that had been heat inactivated in a boiling water bath for 7 min were made up to a final volume of 125 ml in water or buffer.

Analysis of Headspace Volatiles. After incubation coded samples were submitted to sensory evaluation, initially on an informal basis with small groups of three to five trained personnel. Judges were asked if they could detect differences in aroma, to describe these differences, and in particular to determine which samples had a characteristic raw carrot aroma. In some instances samples that appeared active were further compared with the various controls in triangular tests involving 10 judges.

Volatiles present in the enzyme reaction mixtures and controls were isolated and concentrated using a rapid method developed by modifying the gas-entrainment, on-column trapping technique described by Morgan and Day (1965). The method and its use in conjunction with gas-liquid chromatography-mass spectrometry (glc-ms) for identifying volatiles present in aqueous carrot extracts is described in detail elsewhere (Heatherbell *et al.*, 1970; Heatherbell *et al.*, 1970; Heatherbell and Wrolstad, 1970).

RESULTS AND DISCUSSION

Preliminary experiments confirmed the "flavorese" concept in that it was possible to enzymatically regenerate a fresh, raw carrot aroma in commercial dehydrated carrots. Dehydrated carrots however were an undesirable form of "precursor" owing to a strong, caramelized odor that tended to mask the more subtle raw carrot aroma induced enzymatically, and various deodorized sources of "precursor" (substrate) were investigated. Early experiments involved the use of 4 g of reconstituted freeze dried precursor incubated for 90 min at 37° C with 50 mg of acetone powder A, followed by organoleptic evaluation. At the levels of enzyme used, the enzyme-only controls were odorless and did not contribute a background aroma. The precursor-only control was also essentially odorless, but had a bland "hay-like" aroma. Characteristic raw carrot aromas were only developed in the enzyme-precursor samples. Panel member descriptions included "carroty," "fresher," and "raw carrot." These studies indicated it was only necessary to prepare the enzyme-precursor and precursor-only samples. When judges were presented with three coded samples in triangular tests, one of enzyme-precursor and two of precursor-only, in 90% or greater of the cases the enzyme treated sample was detected as different and described as carrot. The fact that precursor treated with heat inactivated enzyme did not develop a raw carrot aroma in any instance is regarded as evidence that the process is enzymatic in nature. However, not all experiments resulted in flavor regeneration. Acetone powders prepared by the same method as described for powder A were "active" sometimes and only weakly active or inactive in other instances. In an attempt to obtain enzyme extracts that were reproducibly active, several methods of enzyme preparation were investigated. As enzyme preparations that were essentially odorless (to minimize background interference) and in a form convenient for storage were desirable, the preparation of acetone powders was preferred. Cell-free extracts investigated had background carrot aromas and were not suitable. Aqueous carrot extracts were observed to undergo "browning," indicating oxidation of phenolic compounds. As phenolics are reported to interfere with the isolation of plant enzymes and to inhibit their activity (Loomis, 1968), enzyme preparation methods were designed to minimize or prevent this. These included the use of PVP (Loomis and Battaile, 1966), PEG, and direct acetone extraction (Loomis,

1968). However, these acetone powders when reacted with precursor gave similar results to those obtained by using the regular acetone powder (A). Reactions were carried out at pH's ranging from 5–8, and in the presence of 0.01 *M* ethylenediaminetetraacetic acid (EDTA) with no noticeable improvement in the results. An enzyme preparation prepared by ammonium sulfate precipitation using the method described by Manners and Rowe (1968) was inactive. Three different substrate sources were examined, freeze dried precursor, nitrogen purged precursor, and a cooked carrot precursor preparation. Based on sensory evaluation, freeze dried precursor prepared with a 3 hr distillation under reduced pressure was the most active substrate, followed by the nitrogen purged precursor and freeze dried precursor prepared by an exhaustive distillation for 8 hr. The cooked precursor was inactive. With the exception of the ammonium sulfate preparation, all the enzyme preparations were capable of erratic "flavorese" activity, but no preparation was found to be consistently active. Based on sensory analysis acetone powders, (B) produced the best activity, followed by powders (A), (D), and (C) in descending order of activity. Other workers have reported variations in and loss of enzyme activity. Miller (1958) reported erratic enzyme activity in the enzymatic regeneration of pea and bean aroma. No correlation was found between methods of enzyme preparation and enzyme activity. Repeated tests with the same enzyme preparations gave good activity (evaluated organoleptically) on one occasion and little or none on others. Variation in enzyme activity with crude enzyme preparations from tomatoes (Yu *et al.*, 1968b) and oranges (Attaway and Metcalf, 1966) has also been reported. Konigsbacher and Hewitt (1964) used enzyme extracts to develop orange blossom aroma from an odorless orange blossom precursor preparation; enzyme preparations displayed erratic activity and only produced the orange blossom aroma at a pH of 4.

The volatiles present in enzyme reaction samples possessing regenerated carrot aroma and in control samples were examined by glc-ms. Compounds identified are the same as those reported previously as being present in aqueous carrot extracts (Heatherbell *et al.*, 1970). Figure 1 is typical of the results obtained using freeze dried "precursor" (prepared with 8 hr distillation) and acetone powder (B). A 3:1 effluent splitter permitted simultaneous odor evaluation of the peaks recorded. The enzyme-only control is not included, since apart from the presence of an acetone peak it had no detectable background. The substrate-only chromatogram shows the extent to which volatiles are removed with reduced pressure steam distillation (for 8 hr) followed by freeze drying. The only major difference between the two chromatograms is the presence of a background acetone peak (peak no. 4) contributed by the acetone powder, appearing in the enzyme treated substrate chromatogram. In an attempt to exclude the possibility of enzyme induced peak(s) being present but masked by one of the background peaks, the separation of compounds was examined on three different columns, Carbowax 20M, SF96-50, and 3-tris-(2-cyanoethoxy)propane (TRIS). In each instance, enzyme reaction samples and duplicate controls were examined. The appearance of new enzyme induced compound(s) and/or significant increases in existing compounds, coinciding with the regeneration of raw carrot aroma, was not detected. As the possibility of a masked peak still existed, attempts at preparing an active substrate with essentially no detectable background were investigated. A nitrogen purged precursor material, prepared as described in the experimental section, was the closest to fulfilling these needs. Figure 2 illustrates the extent to which the background was reduced. Essentially only the

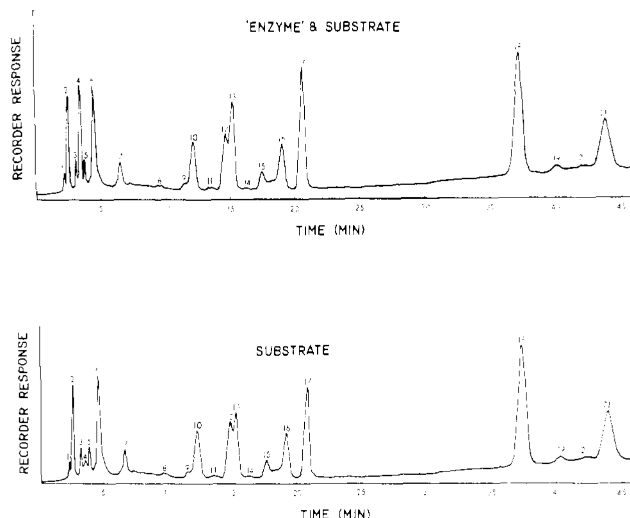

Figure 1. Analysis of the headspace volatiles present in substrate (vacuum distilled for 8 hr, freeze dried) and enzyme treated substrate samples, using an SF96-50 column. Peak no. 2, acetaldehyde; 4, acetone; 6, ethanol; 10, α-pinene; 12, β-pinene and sabinene; 13, myrcene; 15, limonene; 16, γ-terpinene; 17, terpinolene; 18, caryophyllene; 21, γ-bisabolene

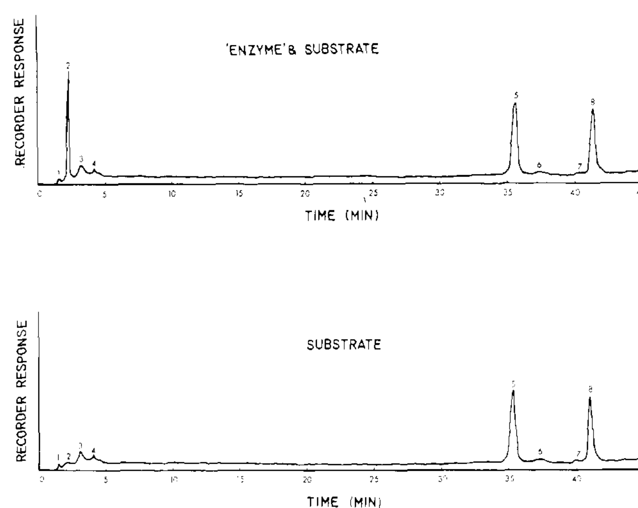

Figure 2. Analysis of the headspace volatiles present in substrate (nitrogen purged) and enzyme treated substrate samples, using a Carbowax 20 M column. Peak no. 1, acetaldehyde; 2, acetone; 3, ethanol; 4, α-pinene; 5, caryophyllene; 8, γ-bisabolene

higher boiling compounds that were originally present in large amounts (*e.g.*, peak no. 5, caryophyllene, and peak no. 8, β-bisabolene), remain. However, the enzyme treated substrate was devoid of any new peaks or increases in existing peaks. These results would indicate that the compound or compounds responsible for carrot aroma are beyond the limits of detection of the method. This would not be unreasonable, because several compounds important in flavor have been reported with aroma thresholds well beyond the limits of instrumental detection. For example, 2-methoxy-3-isobutylpyrazine has a bell pepper aroma at concentrations of a few parts per trillion (Buttery *et al.*, 1969). Also, although the headspace analysis system used did provide a good recovery of a wide range of compounds, if the compound responsible for carrot aroma was very high boiling, and particularly if it were polar, it may not have been recovered from solution by this method.

A freeze dried precursor sample was also prepared using the

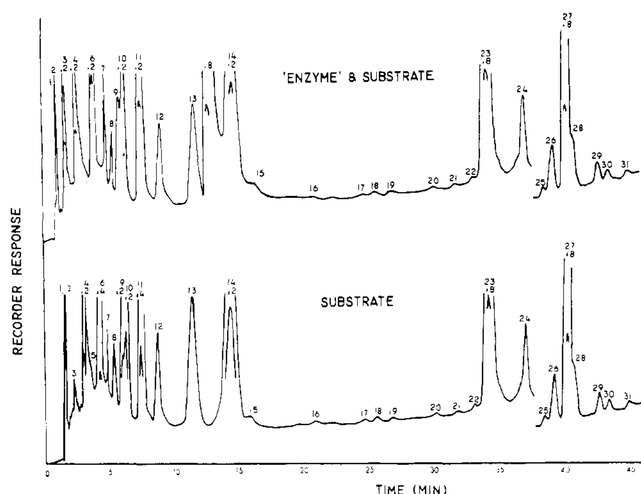

Figure 3. Analysis of the headspace volatiles present in substrate (vacuum distilled for 3 hr, freeze dried) and enzyme treated substrate samples, using a Carbowax 20 M column

less exhaustive reduced pressure steam distillation conditions of 3 hr at approximately 0.5 mm pressure. This substrate, upon reconstitution, did possess a weak carrot-like aroma and was therefore not as good a control. The background (Figure 3) is several fold that obtained by distilling for 8 hr. However, when treated with suitable enzyme preparations, a stronger characteristic raw carrot aroma than achieved with the other freeze dried substrate was induced. Apart from the appearance of a new peak (between γ-terpinene, peak no. 13 and terpinolene, peak no 14) having a retention time and odor corresponding to *p*-cymene, the substrate and enzyme treated substrate chromatograms are essentially identical. *p*-Cymene is readily formed by isomerization of other monoterpene hydrocarbons (Wrolstad and Jennings, 1965); however, a corresponding decrease in other monoterpenes was not apparent. The possibility also exists that the compound from which *p*-cymene was being formed was so unstable and transient under conditions of analysis that it was not ddtected. It is quite possible a compound that is unstable under the conditions of analysis is responsible for fresh carrot aroma. Recently Varo and Heinz (1970) reported a terpene aldehyde, 1,4-*p*-methadiene-7-al, believed important in cumin odor, that was highly reactive and unstable during analysis conditions, undergoing rearrangement and disproportionation reactions to produce the aromatic terpene aldehyde, cumin aldehyde (as well as other related compounds). Although the formation of this new peak was reproduced in three consecutive experiments using the same enzyme (Acetone powder A) and precursor materials, it was not reproduced at any other time during this study using the same or different enzyme and precursor preparations. In these experiments, as in earlier experiments using freeze dried precursor material, the duplicate controls were generally reproducible to $\pm 10\%$. However, periodically the concentration of an individual compound could vary by as much as $\pm 25\%$. Earlier studies on carrot volatiles have indicated that this type of variation could account for the inherent variation of volatiles in the source material (Heatherbell *et al.*, 1970). Because of this, the limits of reproducibility are set at $\pm 25\%$. However, it is possible that enzymatically induced increases in the order of 50% may have occurred for a given compound. Increases of this magnitude for α-pinene, sabinene, myrcene, and terpinolene were frequently observed and may have been real enzyme-induced increases, but owing to the inherent variation in volatile content of the controls, the results must be interpreted with extreme caution. Even if these increases represented enzyme activity, they are not believed to be responsible for the induced carrot aroma, as in some instances the concentration of induced compound was below the reported threshold for detection (Heatherbell *et al.*, 1970) and on several occasions there were no detectable increases of these compounds coinciding with the induced characteristic carrot aroma. The possibility exists that rather than synthesis occurring, an enzymatic or chemical hydrolysis may result in an irregular release of volatiles, for instance by glycosidase action or from polysaccharide encapsulation.

Although the results reported in this study are somewhat inconclusive, they do illustrate the complexity of the system under study. The compound(s) responsible for carrot aroma remains elusive and may be a potent compound present in very low concentrations beyond the limits of detection of the method used, or very labile and unstable under the analysis conditions. Of course, the possibility also exists that carrot aroma results from a complex interaction of several compounds.

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