Acknowledgment

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FLAVOR ENHANCEMENT REVIEW

Enzymatic Enhancement of Flavor

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Flavor enzymes acting on dehydrated cabbage were assayed by paper and gas liquid chromatography and by mass spectrometry. Sensory studies established flavor formation in processed string beans treated with enzymes. Protein fractions from fresh onions were active on S-alkyl cysteine sulfoxide and enhanced the flavor of dried onions. Interaction of an enzyme and substrate derived from raspberries resulted in the simultaneous formation of volatiles and raspberry aroma. The number of flavor enzyme preparations which have been reported indicates these agents may soon find wide application in upgrading the flavor and acceptability of processed foods.

Processing fresh foods often causes loss or unfavorable change in natural flavor. This is particularly noticeable in severely heat-processed foods, such as those which have been dehydrated. This laboratory has been working on the problem of improving the flavor of processed foods by treating them with enzymes. It is the purpose of this article to review this work and that of others which has a bearing on this problem.

Figure 1 illustrates the loss of flavor in a fresh food on processing and the restoration of natural flavor by addition of flavor enzymes. Previous publications (*2–6, 8–10*) have reported that this process for the restoration of natural flavor to processed food has wide application and can be applied to canned, frozen, and dehydrated foods.

The restoration of flavor by enzymes is based upon the concept that the flavor in fresh foods results from the action of enzymes upon substrates or flavor precursors present in the foods. Figure 2 is a flow diagram of natural flavor development, loss in processing, and restoration of natural flavor by enzyme additives.

Enzyme Sources

A commercial process employing flavor enzymes requires an inexpensive enzyme source. As it may be impractical to use the fresh food as the source of enzymes to treat the processed food, other possible enzyme sources have been investigated (*4*). Certain biologically related materials in the Cruciferae family, such as cabbage, mustard, horseradish, watercress, radish, cauliflower, and turnips, have been shown to be an effective enzyme source for the enhancement of the flavor of processed cabbage (*4, 9*). The nonedible part of the string bean plant, i.e., leaves, stems, stalks, and roots, has been reported as an enzyme source for the treatment of dehydrated, frozen, or canned string beans (*2*); microbial materials, such as thioglucosidase from *Aspergillus sydowi*, have been studied as an enzyme source (*4*) to treat processed cabbage (*11*).

It is possible to screen the flavor-enhancing qualities of an enzyme preparation upon a processed food by sensory analysis. The effect of adding flavor-producing enzymes to processed food is easiest to observe where the flavor, especially the aroma factor of the processed food, is low and that of the fresh food is high and distinctive, as in dehydrated cabbage and fresh cabbage, respectively. For sensory analysis, optimum conditions are obtained when processed food is treated to remove all of its flavor, leaving the flavor precursors intact. These preparations are referred to as deodorized flavor precursors. A crude enzyme preparation from any source, as long as it is active and flavorless, may be screened by adding it to a deodorized flavor precursor. The precursor alone and the enzyme alone can be used as controls.

Source, Specificity, and Properties of Flavor Enzymes for Processed Cabbage

To locate an enzyme source other than the fresh food itself, biologically related materials, namely other members of the Cruciferae family, were investigated (*4, 9*). Cruciferae are noted for their pungency, which is largely due to their mustard oil content. This makes it possible to study flavor propagation in this family with ease by sensory analysis.

Studies were limited to cabbage, mustard, horseradish, and watercress.

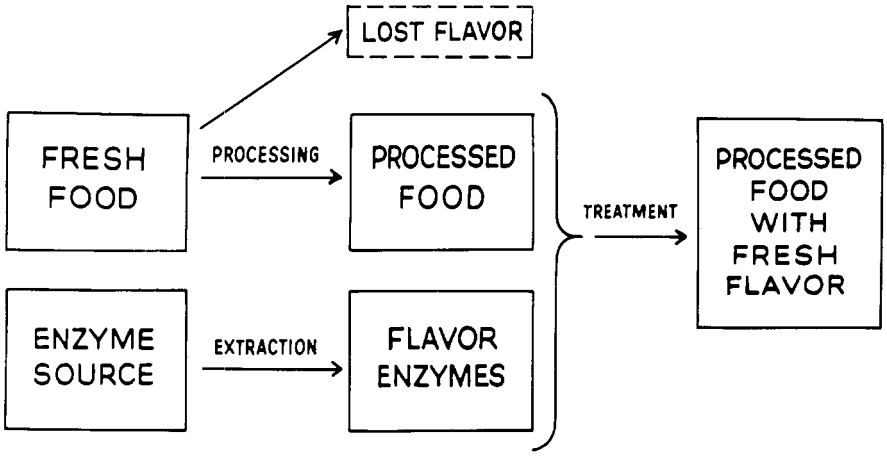

Figure 1. Enhancement of natural flavor of processed food

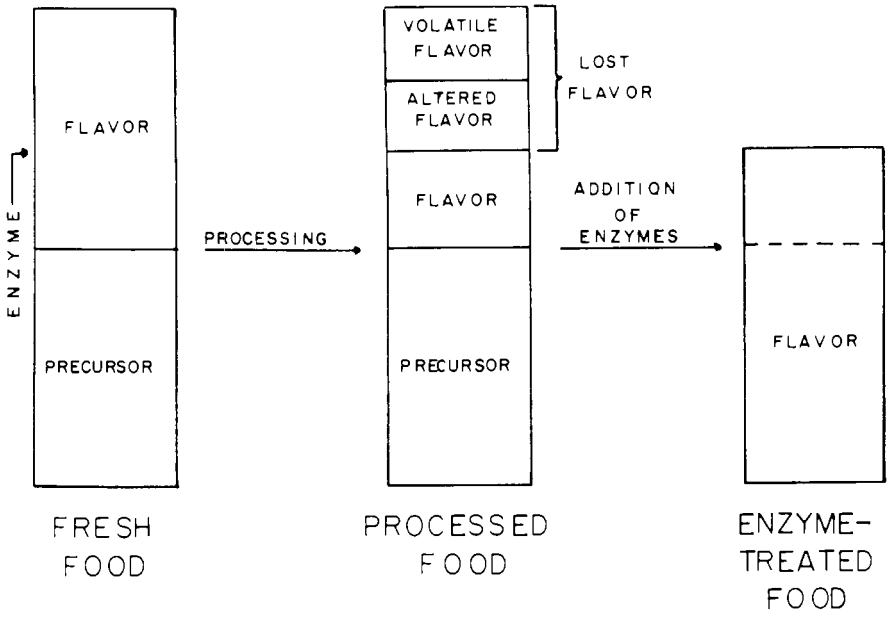

Figure 2. Enzymatic restoration of flavor

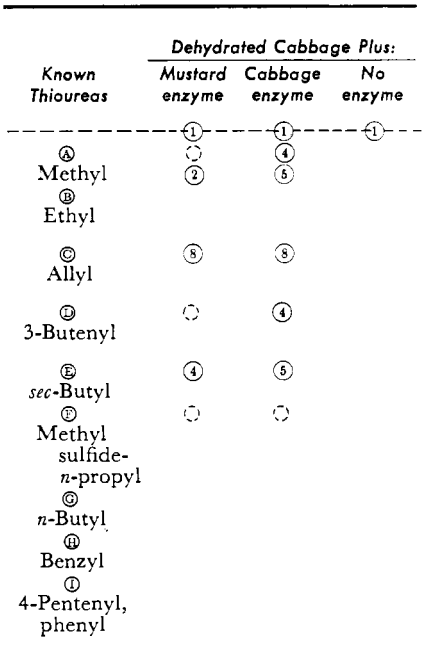

Figure 3. Effect of cabbage and mustard enzymes on dehydrated cabbage

(Numbers refer to relative intensity: 1 = very weak and 9 = very strong; dashed circles indicate compounds seen only when large amounts of thiourea mixtures were chromatographed)

An expert panel found that enzyme preparations prepared from mustard or cabbage yielded somewhat different flavors on the same lot of reconstituted dehydrated cabbage. This was not surprising, since an enzyme often shows variations in activity depending upon the source.

Extension of these criss-cross experiments, where an enzyme from one member of the family is added to a processed food from another member, sometimes led to the development of some novel natural flavors (*4*), and they also served to indicate that the source of the enzyme preparation may be very important for the development of the desired natural flavor. The enzyme preparation made from a member of the same biological family, in some cases, converted the flavor of the processed food into its fresh flavor; in others, it converted the flavor of the processed food into a flavor somewhere between that of the unprocessed food and that of the food from which the enzyme was obtained. Thus, mustard enzyme converts dehydrated watercress into full-flavored watercress, but mustard enzyme bestows a pungency on dehydrated cabbage.

As the sensory panel found a difference in the release of flavor from dehydrated cabbage with mustard and cabbage enzymes, it became important to obtain an objective confirmatory test. This was confirmed by paper chromatographic analysis of the thioureas derived from mustard oils (*9*), according to the method of Kjaer and Rubenstein (*7*).

Cabbage and mustard enzyme preparations (in amounts calculated to contain the same amount of nitrogen) were allowed to react on equal amounts of commercially dehydrated cabbage. These mixtures were then extracted with hexane and treated with concentrated ammonium hydroxide to convert the liberated mustard oils into thioureas.

Figure 3 is a schematic representation of several paper chromatograms of the resultant thioureas. The first column gives the zones of a number of known *N*-substituted thioureas as reference points. The effect of treating dehydrated cabbage with mustard and cabbage enzymes are shown in the second and third columns, respectively. Dehydrated cabbage without added enzyme is given in the last column as a control. The control has only one weak zone at the origin.

Qualitatively, both cabbage and mustard enzyme acted alike. In both cases, the major constituent was due to allyl thiourea (zone C). There were five other components present. The identity of these is uncertain, although butenyl thiourea (zone D), *sec*-butyl thiourea (zone E), and methyl sulfide-*n*-propyl thiourea (zone F), may account for three of these.

Quantitatively, the two enzymes behaved differently. Four of the components located near zones A, B, D, and E were more strongly detected in the cabbage enzyme preparation. Furthermore, when smaller and equal amounts of samples were examined, only three components could be found in the sample treated with mustard enzyme, but five were found in the other sample treated with cabbage enzyme.

The samples of mustard and cabbage enzyme, used in the paper chromatographic study, were compared for

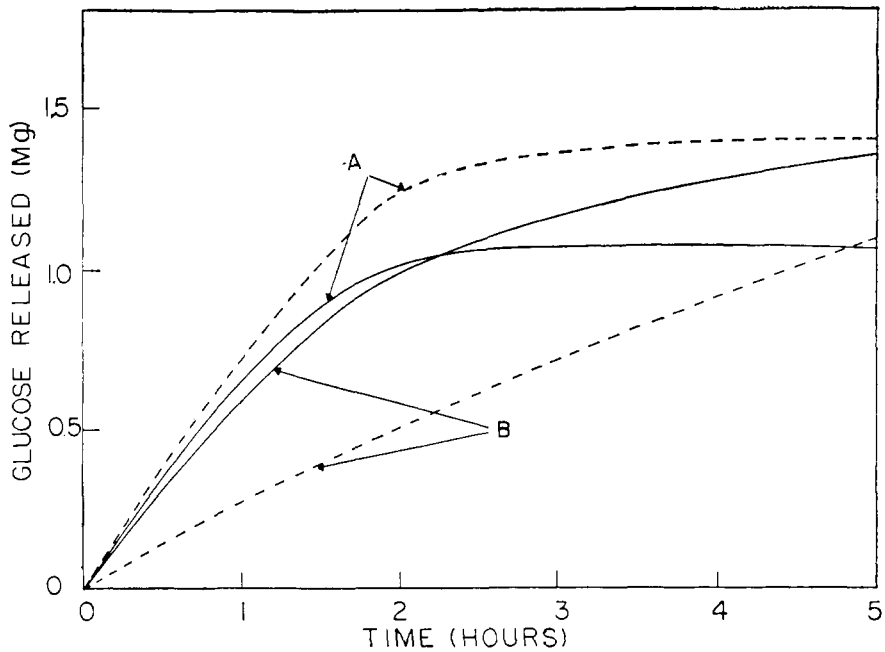

Figure 4. Comparative activity of thioglucosidases from cabbage (A) and mustard (B) on sinigrin (—) and sinalbin(---)

their thioglucosidase activity on sinigrin and sinalbin. These thioglucosides are found in black and white mustard, respectively.

The comparative activity of the thioglucosidases in cabbage and mustard acting on sinigrin and on sinalbin is given in Figure 4. The relative activities of the two enzyme preparations were different. At 1 hour, approximately 0.5 mg. of glucose was formed by the cabbage enzyme acting on sinigrin and on sinalbin, while the mustard enzyme, although it released 0.5 mg. of glucose from sinigrin in one hour, formed only about half as much glucose from sinalbin. The results of the analysis of the two enzyme preparations, acting on dehydrated cabbage, on sinigrin, and on sinalbin, could account for the flavor differences noted in the sensory panel studies. Whether objective analytical data would confirm the subjective sensory observations in other criss-cross experiments in the Cruciferae family is unknown.

Bailey *et al.* (*1*) have recently reported on the composition of the volatile sulfur compounds in fresh cabbage and compared it with that formed by enzyme action on dehydrated cabbage. The analysis was made by gas chromatography and mass spectrometry. The enzyme and dehydrated cabbage preparations were similar to those used by Mackay and Hewitt (*9*), but only the cabbage enzyme was used in this case.

The results, shown in Figure 5, on the enzymatic released volatile isothiocyanates were obtained from gas chromatographic analysis of the volatiles in the head space of fresh, dehydrated, and enzyme-treated dehydrated cabbage. The results are a composite of more than one experiment, and the data can be compared only on a qualitative basis. The fresh cabbage and the enzyme-treated cabbage clearly show the presence of allyl isothiocyanate as the major component and *n*-butyl isothiocyanate as a minor component. The control, consisting of dehydrated cabbage, shows neither isothiocyanate to be present.

Volatile Sulfur Components of Fresh Cabbage, Processed Cabbage, and Processed Cabbage Treated with Flavor Enzymes

The volatile sulfur components of Table I were taken from the data of Bailey *et al.* (*1*). The results of mass spectral and gas chromatographic analysis of fresh and processed cabbage with and without enzymes are shown. The isothiocyanates are similar to those reported by Mackay and Hewitt (*9*).

The source of the sulfides is not known. Some of these compounds may possibly arise from enzyme action on *S*-alkyl-L-cysteine sulfoxides, which have been identified in cabbage (*3*). Sulfoxidases may be present in the crude enzyme preparations used in these studies.

Source, Specificity, and Properties of Flavor Enzymes for Processed String Beans

The source and properties of enzyme preparations which enhance the flavor of canned, dehydrated, and frozen string beans have been investigated by Cort *et al.* (*2*). Several crude enzyme preparations were made from fresh string beans, seeds, sprouts, immature plants, and sun-cured plants remaining at the end of the harvest season. Flavor enzymes were prepared by freezing, grinding, or drying the plant materials to release the enzymes from the cells, and then extracting with tetraborate (pH 7.0) or citrate phosphate (pH 9.3) buffers. The soluble fractions were separated, and the enzyme was precipitated by addition of cold acetone or ethanol. The crude enzyme precipitates were washed, freeze-dried, and weighed. The yield ranged from 2 to 6% of the weight of the starting material.

The flavor enzyme activity of the preparations was determined by adding 50 mg. to 100 grams of cooked frozen string beans. This mixture and control samples of unmixed beans and enzyme preparations were incubated at 37° C. for 1 hour. At the end of this time, samples were submitted to a sensory panel, and those which appeared active were further compared in triangle tests.

Table I. Volatile Sulfur Components of Fresh and Processed Cabbage

Components	*Fresh Cabbage*	*Dehydrated Cabbage*	*Dehydrated Cabbage + Enzyme*
Isothiocyanates			
Methyl	+	0	0
n-Butyl	+	0	+
Butenyl	+	0	+
Allyl	+	0	+
Methylthiopropyl	+	0	0
Sulfides			
Hydrogen	+	0	0
Carbonyl	+	0	+
Dimethyl	+	+	+
Diethyl	+	0	0
Dibutyl	+	0	0
Disulfides			
Carbon	+	0	+
Dimethyl	+	0	+
Methyl ethyl	+	0	0
Diethyl	+	0	0
Ethyl propyl	+	0	0
Dipropyl	+	0	0
Propyl butyl	+	0	0
Propyl allyl	+	0	0
Diallyl	+	0	0
Trisulfides			
Dimethyl	+	0	0
TENTATIVE (because of lack of reference spectra)			
Isothiocyanates			
Methylthiomethyl	+	0	0
Methylthiobutyl	+	0	0
Trisulfides			
Diethyl	+	0	0

The sensory assays showed that the crude enzymes, prepared from string beans and mature bean plants, restored the fresh string bean flavor and odor to the cooked frozen string beans. Similar experiments with canned and dried beans produced the same results.

Several samples of flavor enzymes obtained from string beans were assayed for lipase, glucosidase, peroxidase, and alcohol dehydrogenase (ADH) activity. The samples showed only phosphatase, peroxidase, and ADH activity. Peroxidase activity was established with *o*-phenylene diamine and phosphatase by *p*-nitrophenol formation from *p*-nitrophenyl phosphate disodium salt. ADH activity was measured in Thunberg tubes containing 3.2 mg. diphosphopyridine nucleotide (DPN), 1.0 ml. of methylene blue (1:10,000), 0.1 ml. of alcohol under test, 0.7 ml. of heated string bean homogenate, and 30 mg. of flavor enzyme added to the side arm of the Thunberg tube. The volume of this mixture was then adjusted to 3.0 ml. with pyrophosphate buffer (pH 9.2). Air was swept out of the tubes with nitrogen, the tubes were incubated at 37° C., the contents of the side arm tipped in, and the time necessary for decolorization of the methylene blue was recorded. Oxidation of 2-hexenol required 20 minutes under these conditions.

Studies on the composition of the volatile constituents of string beans by Mackay *et al.* (*10*) revealed the presence of six-carbon alcohols and aldehydes, including 2-hexenal. Of these, 2-hexenal appeared important from the viewpoint of flavor. Therefore, it became apparent that only the ADH was important of the three enzyme systems detected in the active flavor enzyme preparations of the string bean. It alone could give rise to the formation of 2-hexenal.

The ADH activity of the enzyme preparations was therefore further investigated, using Thunberg tubes as previously described, with various combinations of alcohols, cofactors, and thiosemicarbazide as a trapping agent for the hexenal. The alcohols included ethanol, 3-hexenol, 2-hexenol, and the cofactors DPN, TPN, cocarboxylase, and heated string bean homogenate at a level of 0.3 ml. Like other ADH enzyme systems, the string bean enzyme complex required cofactors. Ethanol was oxidized in 12 minutes by the string bean enzyme requiring only DPN as a cofactor. However, no oxidation of 3-hexenol occurred under the same conditions unless cocarboxylase, thiosemicarbazide, and heated string bean homogenate were also present, and the reaction required 75 minutes. All these additives were also required for oxidation of 2-hexenol by the string bean enzyme in 20 minutes. Therefore, the string bean enzyme required more cofactors to oxidize six-carbon alcohols than it does to oxidize ethanol. However, when triphosphopyridine nucleotide (TPN) was present, no DPN or other cofactors or thiosemicarbazide was required by the string bean enzyme for oxidation of 2-hexenol in 8 minutes. Filtrates of all reaction products not containing thiosemicarbazide formed dinitrophenyl hydrazones with dinitrophenyl hydrazine.

Since the string bean enzyme contained ADH, a crystalline, commercially available ADH yeast enzyme was substituted for the string bean enzyme and its activity studied under the same conditions. It was found that a mixture of DPN and the yeast enzyme did not require other cofactors for oxidation of ethanol (in 10 minutes), 2-hexenol (15 minutes), and 3-hexenol (60 minutes). The yeast enzyme also differed from the string bean enzyme in that it did not produce flavor in processed string beans even when aerated.

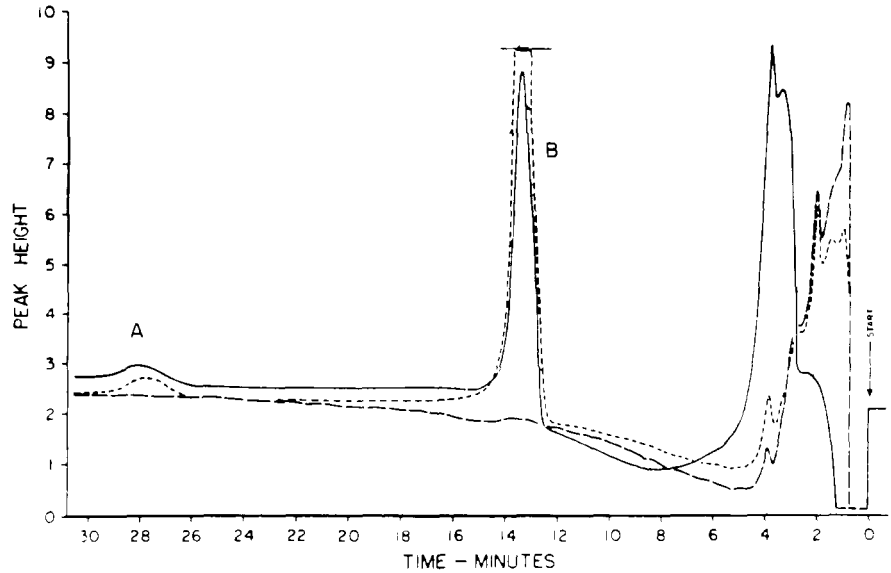

Figure 5. Enzymatic release of isothiocyanates

(*A*, *n*-butyl isothiocyanate; *B*, isothiocyanate)
Sample: 5 ml. vapor
Column: 4 ft. × 4 mm. I.D.
Temperature: 98° C.
Flow Rate: 60 ml./min.
———Fresh cabbage; — — — dehydrated cabbage; - - - - enzyme treated dehydrated cabbage

Table II. Summary of Specificity of String Bean Enzyme

Substrate	*Enzyme*	*Product*
Processed string beans	String bean enzyme (bean or plant)	Enhanced flavor (2-hexenal)
	Commercial peroxidase	No flavor
	Phosphatase of string bean	No flavor
	Commercial alcohol dehydrogenase	No flavor
2-Hexenol	String bean enzyme + TPN	2-Hexenal
	String bean homogenate	2-Hexenal
	Commercial alcohol dehydrogenase + DPN	2-Hexenal

Table II shows a comparison of the flavor-producing properties of the string bean enzyme with other enzyme preparations in the production of string bean flavor and 2-hexenal. Only the string bean enzyme acting on processed string beans enhanced the flavor of the processed product, and, therefore, the source of the string bean enzyme is limited to the beans or the string bean plant. String bean enzyme and commercial alcohol dehydrogenases will produce 2-hexenal from 2-hexenol, but since 2-hexenal is only one component in the volatiles of string beans, it is not adequate to substitute this alcohol dehydrogenase for the string bean enzyme preparation in enhancing the flavor of processed string beans.

Flavor Enzymes of Onions

The literature (*14*) indicates that when an onion is crushed, the resulting pungency is due to the reaction given in Figure 6. The *S*-substituted L-cysteine compounds are converted by

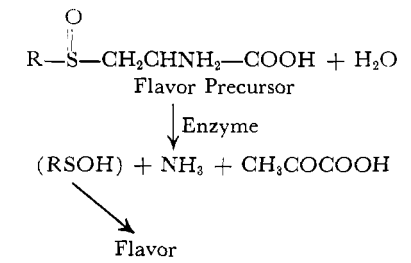

Figure 6. Sulfoxidase action in onion

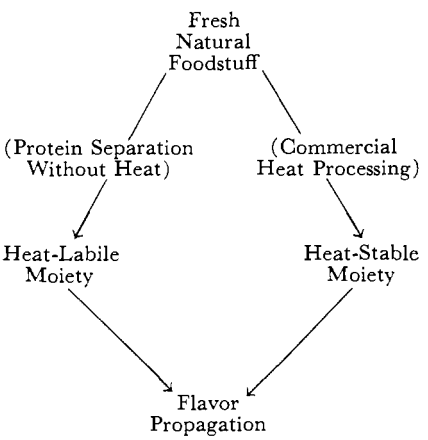

Figure 7. Separation and recombination of heat-labile and heat-stable moieties

the sulfoxidase enzyme present into unstable sulfenic acids (RSOH) and ammonia and pyruvic acid. The hypothetical unstable acids may then undergo spontaneous changes in several ways to form the characteristic flavor of onions.

This laboratory (*4*) has reported the preparation from the soluble fraction of the onion of an active flavor enzyme preparation which will enhance the flavor of a heat-processed onion. More recently, Schwimmer has reported (*14*) the preparation of a partially purified appropriate enzyme preparation from the same source which shows sulfoxidase activity. Others have reported the occurrence of *S*-methyl-L-cysteine sulfoxide and *S*-propyl cysteine sulfoxide in onion (*15*).

Schwimmer (*12*) has studied the stoichiometry of the reaction of *S*-propyl cysteine sulfoxide with sulfoxidase enzyme prepared from onion. He found that as each mole of the substrate disappeared, one mole of ammonia and pyruvate appeared. If a correlation could be established between any of the products of this reaction and onion pungency, it would furnish additional proof that the sulfoxidase-active fraction represents an important part of the flavor enzyme system of the onion. According to Schwimmer's

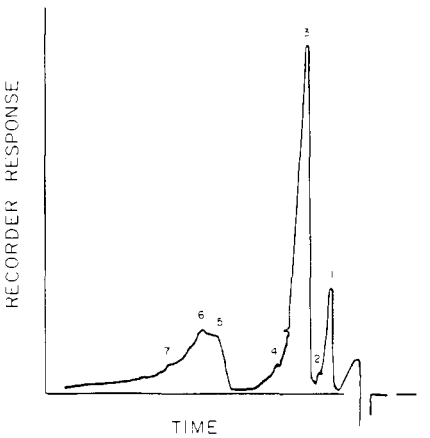

Figure 8. Enzymatic formation of volatile compounds in raspberries

Sample: 2 ml. vapor
Column: 130 × 0.6 cm. I.D.
Temperature: 95° C.
Flow Rate: 93 ml./min.

work (*13*), a correlation does exist between the olfactory threshold concentration of fresh onion juice and the amount of enzymatically developed pyruvic acid in the juice.

Discussion

In seeking sources for flavor enzymes, a methodology or technique has been developed for fractionation of a natural food source into its heat-labile and heat-stable moieties (Figure 7). It results in the preparation of cell-free enzyme and substrate fractions. By recombining them in an environment which encourages enzyme action and following the course of this interaction by flavor development, sources other than the fresh food have been discovered for these enzymes. This has been done by expanding the natural food source to include biologically related materials by criss-cross experiments within the Cruciferae family, or by contracting it to parts of the natural food source, as in the investigation of the edible and nonedible parts of the string bean plant. By introduction of the concept of time, the natural food source may be examined at different stages in its life cycle as a source for flavor enzymes and substrates.

In this manner, the technique has been used to investigate the development of aroma and volatiles in the ripening of fruit. According to Weurman (*16*), an enzyme preparation made from ripe raspberries and a deodorized nonvolatile substrate prepared from unripe raspberries, when mixed under controlled conditions, resulted in the simultaneous formation of raspberry aroma and of a number of volatile compounds.

The formation of these volatiles, which were detected by gas chromatography, as shown in Figure 8, confirms the presence of nonvolatile precursors in the unripe fruit. Seven volatile compounds appear to have been formed, the identities of which are unknown. These may play a significant role in the characteristic aroma of raspberries, since they appear simultaneously with the aroma.

Information was also sought on the nature of the enzymes present in the raspberry by testing enzymes from various sources on the deodorized, nonvolatile substrate. None of the several types of commercially available enzymes, when added to the raspberry substrate, developed any aroma characteristic of the raspberry. This again demonstrates the specificity which exists in flavor or aroma-producing enzymes in foods, and thus limits the sources available for these enzymes.

Conclusions

By relying on our gustatory senses, which respond to flavor, many complex enzyme and precursor systems which interact with the formation of flavor have been proved to exist in natural food sources. These complex enzyme systems have been used in restoring natural flavor in processed foods.

It is also clear from the studies reported here that a basic technique has been developed and utilized for the study of the enzymatic propagation of flavor. The technique utilizes sensory evaluation complemented by evaluation with the accessory techniques of gas and paper chromatography and of mass spectroscopy. As the instrumental analytical methods for the detection of volatiles improves, new enzyme systems will be uncovered, and more detailed studies concerning the enzymatic formation of volatile compounds will be possible. The formation of flavor and aroma is only one of the enzymatic processes which will gain by this advance.

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LACTASE ACTIVITY MEASUREMENT

Evaluation of Lactase Preparations for Use in Breadmaking

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The lactase activity of preparations from yeast, fungal, and bacterial sources was determined by measuring manometrically the carbon dioxide formed by yeast fermentation of enzymatically hydrolyzed lactose. The effects of substrate concentration, temperature, and pH on the activity of lactases from fungal and bacterial sources were studied. The measured response was governed by amount and activity of tested lactases and was essentially linear for the enzyme preparations employed. The procedure was advantageously employed in an evaluation of lactases from various sources from the standpoint of their use in panary fermentation.

TWO PRINCIPAL METHODS are used for determining β-galactosidase (lactase) activity (*3*). In one method, *o*-nitrophenyl-β-galactoside (ONPGal) is used as substrate, and the freed *o*-nitrophenol is determined colorimetrically. This method has been extensively used to determine the β-galactosidase activity in bacterial sources. However, the affinities of enzymes from different sources for the substrate vary considerably (*18*). The affinity of β-galactosidase from *Escherichia coli* for *o*-nitrophenyl-β-galactoside is 10 times as large as for lactose (*6*); for other bacterial sources, it is three times as large (*11*). A commercial fungal lactase (*12*) shows hydrolysis rates of *o*-nitrophenol-galactoside higher than hydrolysis of lactose by a factor of about 100 times. Consequently, the results obtained by action of the enzyme on ONPGal cannot be transposed directly to the rate of hydrolysis of lactose. In the second assay method, lactose is used as substrate and the hydrolysis is measured by one of several techniques. Tauber and Kleiner (*16*) developed a colorimetric method for the determination of monoses in the presence of bioses. However, large amounts of lactose interfered with the determination of the hydrolytic products of lactose. Glucose formed from hydrolysis of lactose also can be determined by a gasometric method employing a Warburg apparatus (*3*).

During a survey of different sources of lactase and their application in bread baking (*10*), the authors deemed it desirable to evaluate the lactase activity of various preparations under conditions compatible with those found during panary fermentation. The method used is based on the fact that *Saccharomyces cerevisiae* neither assimilates nor ferments lactose (*4*). By employing yeast, a buffered nitrogen base medium, and the glucose which is split off from lactose by lactase as the only source of energy for yeast fermentation, the amount of carbon dioxide evolved is measured manometrically, using pressuremeters (*14*).

Experimental

Materials. The six lactases used in this study were: a crude bacterial extract containing 24 mg. protein per ml. (*1*), a lyophilized extract of *E. coli* containing about 5% lactase (*13*), a crude extract of lactase from *E. coli* (*2*), a commercial enzyme preparation from yeast (*9*), and two fungal, A and B, commercial enzyme preparations having optimum activities over pH ranges of 6.0 to 7.0 and 4.5 to 5.5, respectively.

Apparatus. Pressuremeters (*14*) were employed to determine lactase activity. The manometers were filled with ethyl lactate (1.031 grams per ml. at 20° C.) colored with crystal violet (*8, 17*) to provide high sensitivity.

Reagents. Reagent grade lactose, galactose, and glucose (chromatographically pure).

Nitrogen base medium for carbon assimilation tests with yeast prepared in the laboratory according to the formula in the Difco Manual (*5*).

Yeast suspension, 7.5 grams in 95 ml. of water, prepared from Fleischmann's wet cake baker's yeast.

Analytical procedure. Crystalline lactose was weighed in one side of the aluminum cup of the pressuremeter. At 1 minute intervals, 10 ml. of nitrogen base medium, 10 ml. of yeast suspension, 10 ml. of 0.1*M* phosphate buffer solution, aliquots of enzyme suspension, and water to give a total volume of 40 ml. of liquid were added. The contents were mixed well prior to tightening the lids, and the pressuremeters placed in a water bath at 30° C. (except for the series of studies on temperature effect on lactase activity). After 5 minutes, the pressure was released; and after equilibration for 15 minutes, four pressure readings were taken at 30-minute inter-