Kester, J. J.; Fennema, O. R. Edible Films and Coatings: A Review. *Food Technol.* **1986**, *40* (12), 47–59.
Kirchner, J. C.; Miller, J. M. Volatile Water-Soluble and Oil Constituents of Valencia Orange Juice. *J. Agric. Food Chem.* **1957**, *5*, 283.
Lakshminarayana, S.; Luis-Samimento, L.; Isabel-Ortiz, R. J.; Siade, G. Extension of Storage Life of Citrus Fruits by Application of Candelilla Wax Emulsions and Comparison of its Efficiency with Tag and Flavorseal. *Proc. Fla. State Hortic. Soc.* **1974**, *87*, 325–330.
Lawrence, J. F.; Iyengar, J. R. Determination of Paraffin Wax and Mineral Oil on Fresh Fruits and Vegetables by High Temperature Chromatography. *J. Food Saf.* **1983**, *5*, 119.
Motlagh, H. F.; Quantick, P. Effect of Permeable Coatings on the Storage Life of Fruits. I. Pro-long Treatment of Limes (Citrus aurantifolia cv. Persian). *Int. J. Food Sci. Technol.* **1988**, *23*, 99–105.
Nisperos-Carriedo, M. O.; Shaw, P. E. Comparison of volatile flavor components in fresh and processed orange juices. *J. Agric. Food Chem.* **1990**, *38*, 1048–1052.
Paredes-Lopez, O.; Camargo-Rubio, E.; Gallardo-Navarro, Y. Use of Coating of Candelilla Wax for the Preservation of Limes. *J. Sci. Food Agric.* **1974**, *25*, 1207–1210.
Rice, R. G.; Keller, G. J.; Beavens, E. A. Flavor fortification of California frozen orange concentrate. *Food Technol.* **1952**, *6*, 35–39.
Shaw, P. E. Essential Oils. In *Citrus Science and Technology*; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; AVI: Westport, CT, 1977; Vol. 1, pp 463–478.
Shaw, P. E. Review of Quantitative Analyses of Citrus Essential Oils. *J. Agric. Food Chem.* **1979**, *27*, 246–257.
Shaw, P. E. The Flavor of non-alcoholic fruit beverages. In *Food Flavours. Part B. The Flavour of Beverages*; Morton, I. D., Macleod, A. J., Eds.; Elsevier Science: Amsterdam, 1986; pp 337–368.
Smith, S. M.; Stow, J. R. The Potential of a Sucrose Ester Coating Material for Improving the Storage and Shelf-life Quantities of Cox's Orange Pippin Apples. *Ann. Appl. Biol.* **1984**, *104*, 383.
Ukai, N.; Ishibashi, S.; Tsutsumi, T.; Marakami, K. Preservation of Agricultural Products. U.S. Patent 3,997,674, 1976.

Received for review October 6, 1989. Accepted March 5, 1990. Mention of a trademark of proprietary product is for identification only and does not imply a warranty or guarantee of the product by the U.S. Department of Agriculture over other products which may also be suitable.

Registry No. Acetaldehyde, 75-07-0; hexanal, 66-25-1; ethyl acetate, 141-78-6; ethyl butyrate, 105-54-4; methyl butyrate, 623-42-7; methanol, 67-56-1; ethanol, 64-17-5; hexanol, 111-27-3; *cis*-3-hexenol, 928-96-1; isobutyl alcohol, 78-83-1; limonene, 138-86-3; valencene, 4630-07-3; octanal, 124-13-0; *trans*-2-hexenol, 928-95-0; carbon dioxide, 124-38-9; oxygen, 7782-44-7; EC-25, 125622-48-2; TAL Pro-long, 89468-99-5.

Immobilized Endo-β-glucosidase Enriches Flavor of Wine and Passion Fruit Juice

Oded Shoseyov,[†,§] Ben Ami Bravdo,[*,†] Dan Siegel,[†] Alexander Goldman,[∥] Shlomo Cohen,[⊥] Lisa Shoseyov,[†] and Raphael Ikan[#]

Department of Horticulture, Faculty of Agriculture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot, Israel, Department of Organic Chemistry, Faculty of Natural Sciences, The Hebrew University of Jerusalem, Givat Ram Jerusalem, Israel, and Israel Wine Institute, Rehovot, Israel

Aspergillus niger endo-β-glucosidase was immobilized to acrylic beads as well as to corn stover cellulose particles. The efficiency of the immobilization at pH 6.5 to the acrylic beads, with respect to activity, was higher than that to the cellulose but still low (10%). However, higher immobilization efficiency for cellulose (30%) was achieved at pH 4.5. The thermal stability of the enzyme was improved by its immobilization in both methods. Free and immobilized endo-β-glucosidases were used to treat Muscat Roy wine and passion fruit juice (pH 2.45). GC–MS analysis as well as sensory evaluation indicated a significant increase in flavor compounds, monoterpene alcohols, and linalool oxides in the wine and linalool, benzyl alcohol, and benzaldehyde in the passion fruit juice. The precursor of the benzaldehyde is a cyanogenic glycoside as evident from the evolution of cyanide followed by the enzymatic treatment.

Enzymatic hydrolysis of flavor precursors attracts many researchers (Schreier, 1988). Cordonnier and Bayonove (1974) were the first to suggest that must of Muscat of Alexandria (*Vitis vinifera* L.) contains nonvolatile (flavorless) forms of monoterpene glycosides. Williams et al. (1982) identified a series of glycosides, such as β-rutinosides (6-*O*-α-rhamnopyranosyl-β-D-glucopyranosides) and 6-*O*-α-arabinofuranosyl-β-D-glucopyranosides of monoterpene alcohols and β-glycosides of benzyl alcohol and 2-phenylethanol. The glycosides were found in must as well as in wine, indicating that there is no naturally occurring enzymatic hydrolysis during the fermentation that can hydrolyze the glycosides. The importance of the monoterpenes to must and wine flavor has been extensively reviewed (Marais, 1983; Rapp et al., 1984; Strauss et al., 1986). Many fruits, such as passion fruit (Engel and Tressl, 1988), prunes (Chet et al., 1986), apples

* To whom correspondence should be addressed.
† Department of Horticulture, The Hebrew University of Jerusalem.
§ Present address: Department of Biochemistry and Biophysics, University of California at Davis, Davis, CA 95616.
∥ Deceased 1988.
⊥ Israel Wine Institute.
Department of Organic Chemistry, The Hebrew University of Jerusalem.

0021-8561/90/1438-1387$02.50/0

(Schwab and Schreier, 1988), and others, contain glycosides of monoterpenes, C_{13} norisoterpenoids, sesquiterpenes, and other alcohols, possessing a great potential that can be realized by a suitable enzyme. Aryan et al. (1987) studied the properties of different glucosidases in an attempt to find an enzyme capable of hydrolyzing glycosides efficiently under must or wine conditions. They concluded that none of the commercially available enzymes as well as enzymes produced in their laboratory had the desired properties. Recently, we have isolated a unique strain of *Aspergillus niger* that secretes an endo-β-glucosidase (Shoseyov et al., 1988). The enzyme has unique properties such as low optimal pH activity, high K_I (glucose) value [calculated according to the method of Dixon and Webb (1979)], and enhanced activity in the presence of ethanol.

The advantage of using immobilized enzymes over traditional batchwise treatment for industrial processes is primarily due to better control of the enzymatic process. Cuatrecasas (1970) described different methods for immobilization. Schwab and Schreier (1988) reported on simultaneous enzyme catalysis extraction of apple fruit glycosides. Their data indicate that the different glycosides are being hydrolyzed at varying rates. The ramification of the latter is that the flavor profile of a few products (fruit juice, wine, etc.) treated by enzyme varies with the incubation time. Therefore, a fine control of the enzymatic treatment is essential for optimal results.

This paper presents methods for the immobilization of *A. niger* endo-β-glucosidase to synthetic acrylic beads as well as to corn stover particles (natural cellulose), evaluates their efficiency, and demonstrates the potential use of the immobilized enzyme for flavor enrichment of wine and passion fruit juice.

EXPERIMENTAL PROCEDURES

Plant Materials. Yellow passion fruits were obtained from the farm of the Faculty of Agriculture in Rehovot. The juice (pH 2.4, 10° Brix) was filtered through a cheesecloth and used immediately afterward with or without addition of 110 ppm SO_2. Muscat Roy wine (pH 3.2, 12% alcohol) was obtained from the Israel Wine Institute. Fresh corn was obtained from the local market; the stover was dried at 110 °C and then ground and fractionated with 500- and 1000-μm screens.

Enzyme Preparation and Assay. *A. niger* endo-β-glucosidase preparation and isolation were done as described elsewhere (Shoseyov et al., 1988). Enzyme activity was measured by using *p*-nitrophenyl β-D-glucopyranoside (PNPG, Sigma). A typical reaction mixture contained 1 mM PNPG in 20 mM citrate buffer, pH 3.4, and the appropriate amount of free or immobilized enzyme. After a 5-min incubation at 35 °C, the reaction was terminated by adding 1 M Na_2CO_3 (200 μL to 1 mL), and OD at 400 nm was measured. Protein concentration was determined according to the method of Bradford (1976) using Bio-Rad protein assay kit. Michaelis–Menten kinetic constants were determined by Lineweaver–Burk plot (Dixon and Webb, 1979).

Enzyme Immobilization. Endo-β-glucosidase was immobilized by using three methods:

1. Immobilization to Oxiran Acrylic Beads (Polexyzyme O., Rohm). Forty milligrams of protein was dissolved in 10 mL of 1 M K_2HPO_4 buffer, pH 6.5. One gram of beads was added, and the slurry was agitated for 48 h at room temperature. The beads were washed sequentially with 50 mL of H_2O (2×), 150 mL of 0.5 M NaCl (3×), and finally 50 mL (2×) of 20 mM citrate buffer, pH 3.4. The immobilized enzyme was kept at 4 °C.

2. Immobilization to Corn Stover Cellulose by Glutardialdehyde (Vallat et al., 1985). One gram of cellulose particles (500–1000-μm fraction) was oxidized by 20 mL of 0.2 M $NaIO_4$ solution, agitated 24 h in the dark. The solution was decanted, and 20 mL of 2.7 M ethylenediamine was added to the cellulose pellet. After 24 h of agitation, the solution was decanted and 20 mL of sodium cyanoborohydride (5 g/L in 50 mM phosphate buffer, pH 6.5) was added to the pellet. After 3 h of agitation, the solution was decanted and 20 mL of 3% glutardialdehyde (in 50 mM pyrophosphate buffer, pH 6.5) solution was added to the pellet. The slurry was agitated for 24 h, and the cellulose particles were washed with 20 mL (2×) of 20 mM citrate buffer (pH 6.5) and then agitated for 24 h with 40 mg of protein. Washing and storage were the same as for the acrylic beads.

3. Immobilization to Corn Stover Cellulose by Acid Chloride Cellulose (Kumar et al., 1986). Sodium periodate oxidation was performed as above to give poly(2,3-cellulosedialdehyde). The particles were further oxidized to give the corresponding carboxyl by 50 mL of 200 mM freshly prepared $NaClO_2$ solution (1.8 g of $NaClO_2$ was dissolved in 75 mL of H_2O, and then 20 mL of 5 M acetic acid was added and the volume was made up to 100 mL). The reaction mixture was incubated at room temperature for 72 h; 50 mL of 100 mM ice-cold HCl solution was added, and the mixture was put on ice for 30 min. The particles were filtered and washed thoroughly with ice-cold water and then freeze-dried. One gram of dry particles was taken in a round-bottom flask equipped with a water condenser and $CaCl_2$ trap. One milliliter of distilled $SOCl_2$ was added, and the mixture was heated until reflux. After 1 h, the flask was cooled and the remaining $SOCl_2$ was vacuum distilled off by using several 5-mL portions of benzene. Twenty milliliters of 50 mM acetate buffer (pH 6.5) containing 40 mg of protein was added to the activated cellulose particles, and the mixture was agitated at 4 °C for 4 h. Washings were conducted as above. Optimizing the pH of the coupling step was done in the same manner except for pH, which varied from 3.5 to 7.5.

Enzymatic Treatment and Flavor Extraction. Two grams of acrylic beads (endo-β-glucosidase immobilized) was added to 1 L of Muscat Roy wine. The flask was sealed under nitrogen and agitated for 72 h at 25 °C. Passion fruit juice with and without 100 ppm SO_2 was treated with 20 ppm endo-β-glucosidase for 24 h at 25 °C. Both the wine and the passion fruit juices were extracted with trichlorofluoromethane (Freon 11) according to the method of Rapp et al. (1976).

Gas Chromatography–Mass Spectrometry. Freon 11 extracts of 250-mL samples were concentrated to 100 μL, and 1-μL samples were injected to a Finnigan MAT 4600 GC–MS or to a GC Varian 3700, both equipped with either 60 m, 0.32 mm i.d. Supelcowax-10 (Supelco) or DB-5 (J&W) capillary columns. Helium was used as a carrier gas, and the oven was programmed as follows: 5 min at 60 °C with gradual heating to 190 °C at 2 °C/min. All peak identifications were confirmed by injecting authentic standards.

HCN Analysis. Five-milliliter samples of passion fruit juice with and without enzyme were placed in 20-mL air-sealed vials. Whatman No. 1 filter paper strips (2 cm long; previously dipped in alkaline solution of 1% picric acid and dried) were placed in the vials above the liquid, and the vials were incubated at 25 °C for 24 h. HCN gas was detected (Kingsburg, 1964) as evident by the color change of the strips from yellow to red.

Sensory Evaluation. Duo trio experiments were conducted by 11 judges, and the significance of the results was determined according to the method of Amerine and Roessler (1983).

RESULTS AND DISCUSSION

Three immobilization methods were employed for the endo-β-glucosidase. The oxiran acrylic beads (Polexyzyme) immobilized 82% of the protein compared to only 5.4% for the acid chloride cellulose matrix when pH 6.5 was used at the coupling step. The immobilization using glutardialdehyde was found to be very inefficient under these conditions (Table I). The relatively low activity remaining on the acrylic beads can be explained by either the "overcrowding" of the protein on the matrix that blocked active sites or preferential coupling of the protein at sites that inactivated it. The K_M value (which remained the same) indicates that the conformation of the active site remains intact. However, to obtain better immobilization efficiencies, several parameters at the

Table I. Effect of the Method of Immobilization on Its Efficiency and the Physicochemical Properties of the Immobilized Endo-β-glucosidase

property	free	Polexy-zyme	corn stover[a] A	corn stover[a] B
protein immobilization, %		82	<1	5.4
activity immobilization, %		10	<1	5.4
K_M, mM	1.0	1.0	0.42	1.0
V_{max}, U[b]/mg of protein	0.22	0.16	0.11	1.67
activity after 2 h at 60 °C, %	50	100		100
optimal pH activity, pH	3.4	3.4		3.4

[a] A, immobilization using glutardialdehyde. B, immobilization using acid chloride cellulose. [b] U, micromoles of *p*-nitrophenol/minute. *p*-Nitrophenyl β-D-glucopyranoside as substrate.

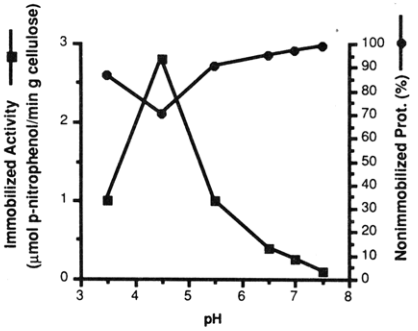

Figure 1. Effect of pH on coupling efficiency of endo-β-glucosidase to acid chloride cellulose.

coupling step have yet to be optimized, such as protein concentration, pH, and temperature. The low protein immobilization efficiency to the acid chloride cellulose at pH 6.5 encouraged us to test different pH values at the coupling step. The results shown in Figure 1 indicate that pH 4.5 should be the pH of choice for immobilizing the endo-β-glucosidase to acid chloride cellulose. It also shows that when optimal conditions for nucleophilic attack are considered, the predicted more alkaline pH should not be the exclusive factor weighed. In fact, the only way to determine the optimal conditions is experimentally, since conformational changes of the protein at different pH values can affect its binding efficiency as well as the activity of the enzyme. Similar results were reported by Kumar et al. (1986) for carboxypeptidase. Analysis of the curve representing the unimmobilized protein (Figure 1) suggests that low binding at suboptimal pH values was the cause of low immobilized enzyme activity rather than a loss of enzyme-specific activity.

Muscat Roy wine was treated with the immobilized enzyme. GC–MS analysis of the treated wine compared to the control indicates that the enzyme treatment increased the concentrations of free monoterpene alcohols such as linalool, terpinen-4-ol, α-terpineol, geraniol, nerol, and *cis*-furan–linalool oxide as well as unidentified peaks such as peak 1 (Figure 2). Note that Figure 1 is somehow misleading since the amount of sample A compared to sample B that entered the column was double, as evident by the internal standard peak. However, the integration data of the gas chromatograms confirmed our observations. Sensory evaluation of the treated wine compared to the control was conducted. Nine of 11 judges identified correctly the treated wines ($p = 0.05$). We found a significant increase in the flavor of the treated wine and a more pronounced Muscat flavor. The appearance of the wine was unaffected by the enzymatic treatment. The activity of the immobilized endo-β-glucosidase that was filtered out of the wine was fully retained. Passion fruit juice was enzymatically treated in the presence or absence of 110 ppm SO_2 (Figure 3). Juice without SO_2

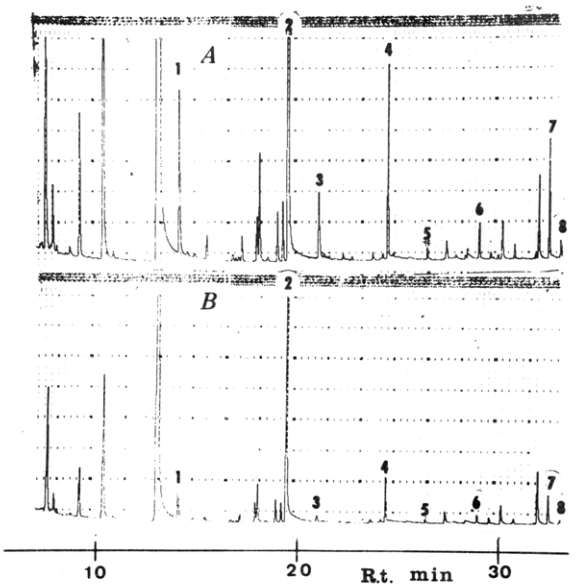

Figure 2. GC analysis of Freon 11 extracts of Muscat Roy wine treated (A) by immobilized endo-β-glucosidase. (B) Control. (2) Internal standard (3-octanol); (3) *cis*-furan–linalool oxide; (4) linalool; (5) terpinen-4-ol; (6) α-terpeneol; (7) nerol; (8) geraniol (Supelcowax-10 column).

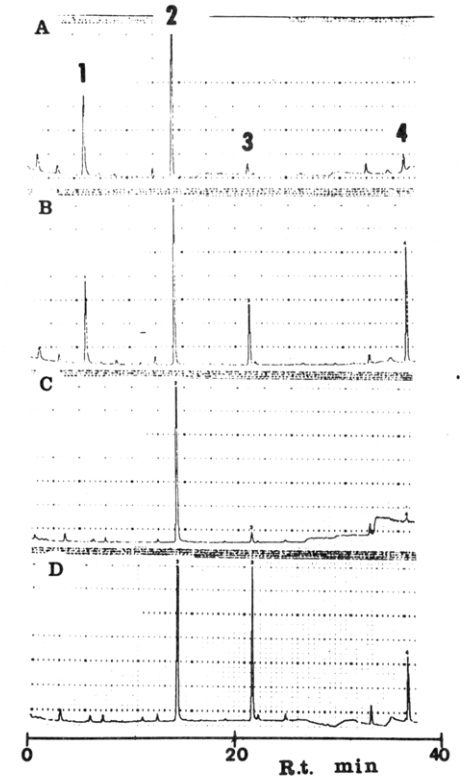

Figure 3. Effect of SO_2 on passion fruit juice, endo-β-glucosidase and the effect of both on the volatiles of the juice after a 24-h incubation at 25 °C. (A) Without SO_2 and enzyme. (B) Without SO_2, with enzyme. (C) With SO_2, without enzyme. (D) With SO_2 and enzyme. (2) Internal standard (3-octanol); (3) benzaldehyde; (4) benzyl alcohol (Supelcowax-10 column).

developed a very strong off-flavor regardless of the presence of the enzyme preparation. It might well be that the evoluation of peak 1 as well as the off-flavor could have been prevented by SO_2 (Figure 3). This could be the result of an oxidative reaction that occurred in spite of the nitrogen presence (see Experimental Procedures). The flavor of the treated passion fruit juice was increased significantly (with and without SO_2) as was evident by the results of a duo trio experiment—11 of 11 judges cor-

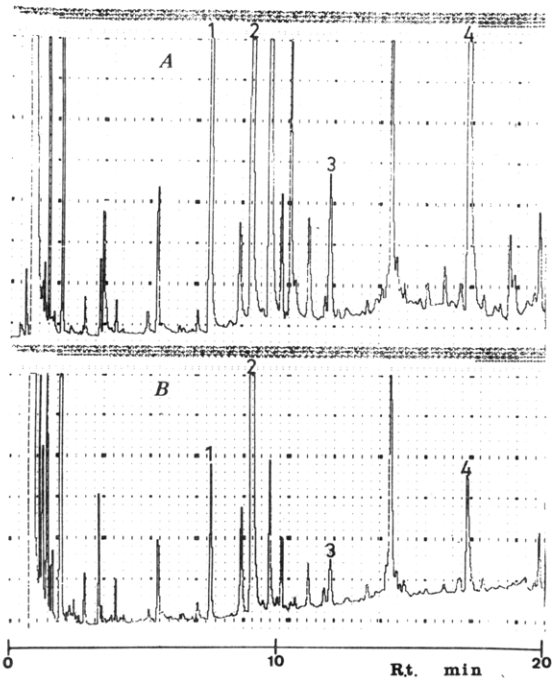

Figure 4. GC analysis of Freon 11 extracts of endo-β-glucosidase treated (A) and control-untreated (B) passion fruit juice. (1) Benzaldehyde; (2) internal standard (3-octanol); (3) linalool; (4) benzyl alcohol (DB-5 column).

rectly identified the treated juice. GC–MS analysis of the treated juice compared to the control indicated a severalfold increase of many volatiles (Figure 4) such as linalool, benzyl alcohol, and benzaldehyde (almond flavor). The appearance of the latter compound was surprising, since normally the hydrolysis of *O*-glycosides results in the formation of sugars and an alcohol. However, cyanogenic glycoside hydrolysis could result in the formation of nitryl and subsequently an aldehyde and HCN, the latter evaporating at low pH. Indeed, we found that the enzymatic hydrolysis was followed by evolution of HCN, coinciding with the results of Conn (1980), who reported the presence of cyanogenic glycosides in *Pasifloraceae*.

In conclusion we suggest that *A. niger* endo-β-glucosidase can be immobilized and used for controlled flavor enrichment of wine and passion fruit juice.

ACKNOWLEDGMENT

We are indebted to Nehama Bar and Marina Charniak for their skilled technical assistance. We thank Prof. M. Wilchek for helping with the immobilization techniques. The research was supported in part by BARD US-1018-85 and the Schoenberger Foundation.

LITERATURE CITED

Amerine, M. A.; Roessler, E. B. *Wines, their sensory evaluation*; Freeman: New York, 1983; 346 pp.

Aryan, A. P.; Wilson, B.; Strauss, C. R.; Williams, P. J. The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes an assessment of possible applications in enology. *Am. J. Enol. Vitic.* **1987**, *38* (3), 182–188.

Bradford, M. A. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

Chen, C. C.; Kuo, M. C.; Liu, S. F.; Wu, C. M. Volatile components of salted and pickled prunes (Prunus mume Sieb et Succ). *J. Agric. Food Chem.* **1986**, *34*, 140–144.

Conn, E. E. Cyanogenic compounds. *Annu. Rev. Plant Physiol.* **1980**, *31*, 433–451.

Cordonnier, R.; Bayonove, C. Mise en evidence dans la boie de raisin var muscat o'alexandria de monoterpenes lies revelables por une ou plusieurs enzymes du fruit. *C. R. Acad. Sci.* **1974**, *278*, 3387–3390.

Cuatrecasas, P. Protein purification by affinity chromatography. *J. Biol. Chem.* **1970**, *245*, 3059–3065.

Dixon, N.; Webb, E. C. *The Enzymes*, 3rd ed.; Longmans: London, 1979; 354 pp.

Engel, K. H.; Tressl, R. Formation of aroma components from nonvolatile precursors in passion fruit. *J. Agric. Food Chem.* **1983**, *31*, 998–1002.

Kingsburg, J. M. *Poisonous plants of the United States and Canada*; Prentice-Hall: Englewood Cliffs, NJ, 1964; 25 pp.

Kumar, S.; Vasudevan, P.; Dua, R. D. Activity of carboxypeptidase a bound to a modified cellulosic matrix. *Appl. Biochem. Biotechnol.* **1986**, *12*, 107–119.

Marais, J. Terpenes in the aroma of grapes and wines: A Review. *S. Afr. Enol. Vitic.* **1983**, *4* (2), 49–60.

Rapp, A.; Hastrich, H.; Engel, L. Gaschromatographisch untersuchungen uber die aroma-stoffe von weinbeeren. I. Anreicherung aund kapillarchromatographische auftrennung. *Vitis* **1976**, *15*, 29–36.

Rapp, A.; Mandery, H.; Gunter, M. Terpene compounds in wine. In *Proceedings of the Alko Symposium on Flavour Research of Alcoholic Beverages*; Nykanen, L., Lehtonen, P., Eds.; FBIFR: Helsinki, 1984; Vol. 3, pp 255–274.

Schreier, P., Ed. *Bioflavour '87*; de Gruyter: Berlin, 1988.

Schwab, W.; Schreier, P. Simultaneous enzyme catalysis extraction: A versatile technique for the study of flavor precursors. *J. Agric. Food Chem.* **1988**, *36*, 1238–1242.

Shoseyov, O.; Bravdo, B. A.; Ikan, R.; Chet, I. Endo-β-glucosidase from *Aspergillus niger* grown on a monoterpene glycoside-containing medium. *Phytochemistry* **1988**, *27*, 1973–1976.

Strauss, C. R.; Wilson, B.; Gooley, P. R.; Williams, R. J. Role of monoterpenes in grape and wine flavor. In *Biogeneration of Aromas*; Parliment, T. H., Croteau, R., Eds.; ACS Sympoisum Series 317; American Chemical Society: Washington, DC, 1986; pp 222–242.

Vallat, I.; Monsan, P.; Riba, J. P. Maltodextrin hydrolysis in fluidized-bead immobilized enzyme reactor. *Biotechnol. Bioeng.* **1986**, *18*, 151–159.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochemistry* **1982**, *21*, 2013–2020.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Glycosides of 2-phenylethanol and benzyl alcohol in *Vitis vinifera* grapes. *Phytochemistry* **1983**, *22*, 2039–2041.

Received for review September 18, 1989. Accepted January 31, 1990.