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Coupled Hydroperoxide Lyase and Alcohol Dehydrogenase for Selective Synthesis of Aldehyde or Alcohol

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Abstract

The main objective of this work was to improve the selective synthesis of a volatile compound: aldehyde or alcohol using a coupled-enzyme system. A novel method of synthesis of C_6-aldehyde or alcohol was carried out in the presence of hydroperoxide lyase (HPLS) activity coupled to alcohol dehydrogenase (ADH) activity. After cleavage of the initial substrate, hydroperoxy fatty acid catalyzed by HPLS, the second enzyme, ADH, can catalyze the reduction of the aldehyde to the corresponding alcohol, or the oxidation of contaminating alcohol into aldehyde, depending on the cofactor present in the medium (oxidized or reduced form). We succeeded in improving the synthesis of one of the products. When coupling HPLS to NADP, the selectivity of hexanal production from 13-hydroperoxy linoleic acid was improved, and hexanol production was reduced 5 to 10 times after 15 min of reaction at 15°C and pH 7.0. In another experiment, HPLS was coupled to ADH in the presence of NADH. The production of alcohol (hexenols) was then favored especially when using 13-hydroperoxy linolenic acid as substrate at concentrations >15 m*M*, reaching 95% of the products. Coupling of the enzymatic reactions (cleavage reduction) not only reduced the number of steps but also allowed us to increase the conversion rate of the initial substrate (hydroperoxy fatty acid). Structures of the compounds produced in this work were confirmed using gas chromatography-mass spectroscopy analysis. Each of these products has its own delicately different fresh odor that can be used in various applications.

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Index Entries: Alcohol dehydrogenase; coupled enzymes; specific synthesis; hexanal; hexanol; hexenal; hexenol; hydroperoxide lyase; hydroperoxy fatty acid.

Introduction

Hydroperoxide lyase (HPLS) and alcohol dehydrogenase (ADH) are two enzymes belonging to the lipoxygenase (LOX) pathway that leads to green notes in plants. The first enzyme cleaves hydroperoxy fatty acids into aldehydes and oxo-acids *(1–5)*. The second enzyme reduces the aldehydes to the corresponding alcohols *(4,5)* (Fig. 1). These volatile products have specific aromatic properties and high added value. Traditional sources such as plant essential oils cannot obtain sufficient quantities of these compounds. This motivated research efforts toward finding alternative natural ways that can satisfy the increasing demand for these materials. The LOX pathway could be used for conversion of fatty acid precursors to the desired volatile products *(6,7)*.

HPLSs are classified into two categories: (1) homolytic HPLSs, generally found in lower plants such as algae and mushrooms, which cleave the link between a carbon-carrying hydroperoxide function and saturated carbon; and (2) heterolytic HPLSs, which cleave the link between hydroperoxide and unsaturated carbon and exist in many plants *(8)*. In 1991, Matsui et al. *(9)* first isolated an HPLS from the membrane fraction from a sheet of tea. HPLS is a heminic protein *(10,11)* and consists of three 55-kDa units. Optimum pH for HPLS activity is between 6.0 and 8.0 *(12)*. The enzyme is generally localized in the chloroplasts associated with thylacoid membrane. The use of detergent is necessary for its recovery. In addition, it can be found in the nonchloroplastic microsomes *(13)*. HPLSs in plants are divided into three classes according to their specificity for the substrate: (1) HPLSs that cleave specifically the 9-hydroperoxy fatty acids, detected in pear *(14,15)*, leading to two C9 fragments; (2) HPLSs specific to 13-hydroperoxy fatty acids, which give C6-aldehyde and C12-oxo-acid, found in tomato fruits *(16)*, tea leaves *(17)*, watermelon seedlings *(18)*, and mint leaves *(19)*; and (3) HPLSs acting at the same time on the 9- and 13-hydroperoxy fatty acids, existing in cucumber *(20)*. The two activities correspond to two isoenzymes that were separated by Mastui et al. *(21)* in 1989.

ADH is the last enzyme involved in the LOX pathway *(4,13,22)*. It is found in plants, animals, and microorganisms. The enzyme converts aldehyde into alcohol and conversely, depending on the presence of cofactor and other effectors in the medium *(23,24)*.

ADH was purified from various plants such as tea, barley and wheat seeds *(25)*, and cucumber fruit *(26)*. However, the detected activity generally does not allow a significant aldehyde reduction. This is owing in part to the difficulty of regeneration of the cofactor (NAD/NADH or NADP/NADPH) crucial for the reaction. A better knowledge of ADH from animals and microorganisms allows its use to obtain products of the LOX pathway. The need for cofactor constitutes a limitation for the reaction because an additional reaction system must be used in the medium. In 1985, Legoy et al.

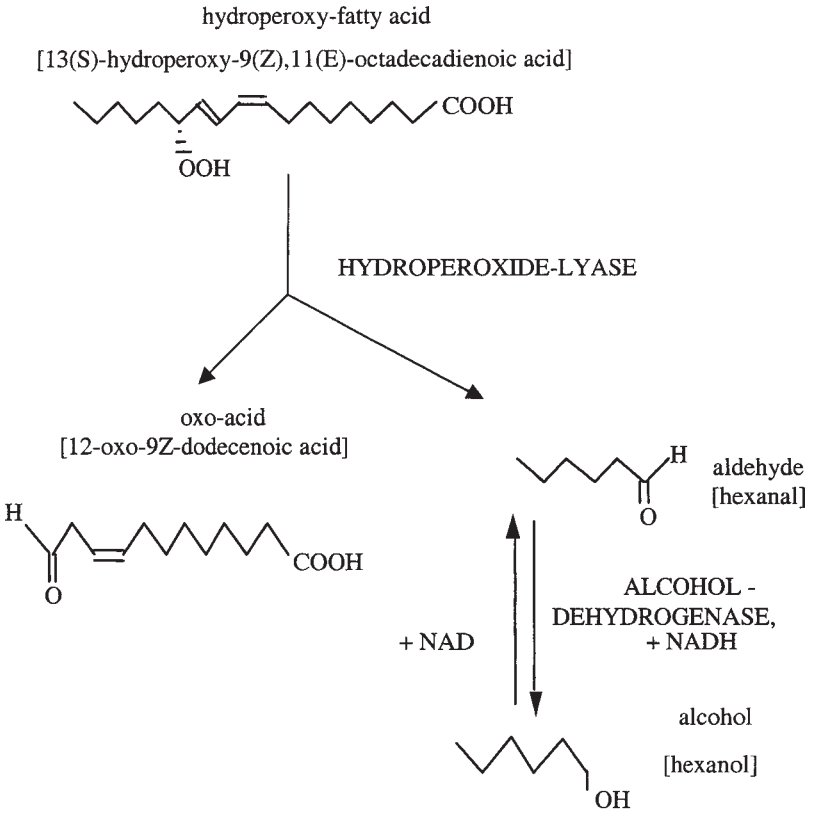

Fig. 1. Reactions of hydroperoxidelyase and alcoholdehydrogenase (ADH) for volatile compound synthesis. 13-Hydroperoxy linoleic acid is an example of initial substrate. ADH reduced aldehyde to alcohol in the presence of reduced nicotinamide adenine dinucleotide (NADH) and oxidized alcohol to aldehyde in the presence of NAD.

(23) studied three systems of regeneration of the cofactor NAD for continuous production of long-chain aldehyde by horse liver ADH: a chemical system in the presence of methosulfate 1-methoxy-phenazin, a coupled-enzyme system with lactate dehydrogenase as the second enzyme, and a coupled-substrate system with acetaldehyde as the second substrate.

Incubation of hydroperoxy fatty acid in the presence of enzyme extract containing HPLS activity in order to produce aldehyde generally does not produce only the target product but also a small quantity of alcohol *(19)*. This is owing to the presence of small quantities of enzyme activities that reduce aldehyde to alcohol, as ADH. To avoid contamination of the product, and to support the selective synthesis of one of the volatile compounds in the system, a novel method was carried out in the presence of various cofactors using two activities—HPLS and ADH—in order to guide the reaction to the production of aldehyde or alcohol. Each of these products has its own delicately different fresh odor that can be used in various applications.

Materials and Methods

Production and Extraction of 13-Hydroperoxy Fatty Acids

Production of 13-hydroperoxy fatty acid from the corresponding polyunsaturated fatty acid was carried out as follows: Glycine buffer, pH 9.0, was mixed with 200 mg of fatty acid (linolenic acid or linoleic acid; Sigma, Paris, France) and 15 mg of soybean LOX (Sigma); and the reaction mixture was kept under stirring with a flow of oxygen under 0.1 bar. The reaction remained for 1 h at 25°C and was followed by withdrawal of samples and measurement of the absorption at 234 nm, corresponding to the production of conjugated double bonds in hydroperoxy fatty acids. These compounds were extracted with diethyl ether (twice the same volume; Sigma), and the organic phase was dried with $MgSO_4$ (Sigma) and then evaporated under vacuum. Hydroperoxide was diluted in ethanol and stored at –20°C.

Extraction of HPLS

The plant material (source of HPLS) was first washed and cut into small pieces. Fifteen grams of plant material (mint leaves and top part of the stems, from a local market) was mixed with 50 mL of 0.2 *M* phosphate buffer, pH 6.0, with 1% Tween-20 added (Sigma). The mixture was homogenized twice during 30 s in a blender and centrifuged for 30 min at 19,500*g* and 4°C. The supernatant was recovered and used as the source of HPLS.

Spectrophotometer Analysis

The reaction of HPLS or HPLS/ADH was directly followed on a spectrophotometer (Beckman DU 530; Paris, France) in a 1.4-mL quartz cell. The medium contained 20 m*M* 13-hydroperoxy linoleic acid as substrate and 0.2 *M* phosphate buffer, pH 6 (as well as 3 U of ADH and 10 m*M* NADH in the coupled-enzyme system), and the reaction was started by adding 0.1 mL of HPLS preparation. Measurement of the loss in absorbance at 234 nm indicated the disappearance of hydroperoxide during the reaction.

ADH activity was tested by measuring the increase in absorbance at 340 nm owing to the apparition of NADH during oxidation at 25°C and pH 8.0 (0.05 *M* Tris-HCl; Normapur, Prolabo). One unit of ADH converts 1 mmol of ethanol into acetaldehyde/min. Horse liver ADH (Sigma) contained 1.5 U/mg and the yeast ADH (Sigma) 300 U/mg.

Reaction of Coupled HPLS/ADH

Reaction of the two enzymes HPLS and ADH was carried out in the same system in the presence of the appropriate cofactor. The bienzymatic system was carried out in 10-mL sealed flasks incubated in rotary shakers at 100 rpm. Substrate and enzyme preparations were mixed in a working volume of 2 mL. One to five millimolar substrate (HPOT or HPOD) was

added to 1.8 mL of mint extract (HPLS) and 3 U of ADH in phosphate buffer, pH 7.0. NAD, NADH, or NADP was used as cofactor in the medium, depending on the target sense of the reaction. The bienzymatic reaction time was 15 min at 15°C.

Gas Chromatography

Volatile compound synthesis was measured by dynamic headspace coupled to gas chromatography (GC). At the end of the reaction, the tube was connected to a Purge & Trap autosampler (Tekmar 2016) and then pressurized with 50 kPa of He. The tube was purged with 40 mL/min of He for 10 min, and the isolated headspace volatile compounds were then adsorbed onto a Tenax trap column (28 cm, Tekmar Concentrator 3000). After removal of water with dry He (50°C, 1 min), the volatile compounds were thermally desorbed at 200°C for 4 min and condensed in a cold trap (MFA 815; Fison, France) maintained at –150°C with liquid nitrogen. Injection into a GC 8000 (Fison) was then done by heating the cryofocusing unit for 90 s at 200°C. Compounds were eluted onto a CP-Wax-57 fused silica capillary column (50 m × 0.32 mm; Chrompack, The Netherlands). Oven temperature was initially 70°C, increased after injection at 13°C/min up to 220°C, and then maintained for 8 min. The carrier gas was He at 1 mL/min, and a flame ionization detector was set at 250°C. Retention times were 6.7 min (hexanal), 7.7 min (3*Z*-hexenal), 8 min (2*E*-hexenal), 9 min (hexanol), and 9.5 min (3*Z*-hexenol). Total hexenal and hexenol concentrations were calculated by considering the sum of the peak areas of the corresponding isomers (2*E* and 3*Z*, if present).

Product Identification

The reaction products were identified by coupling the headspace-GC (Hewlett-Packard 5890) to a quadrupole mass spectroscope (Hewlett-Packard). The He flow rate was 1.2 mL/min. Mass spectrometer (MS) conditions were as follows: mass scan range, *m/e* 20–550; source temperature, 150°C; ionization voltage, 70 eV. NBS 75 K.L library was used to confirm compound identification.

1-Hexanal

1-Hexanal was produced from 13-hydroperoxy linoleic acid in the presence of *Mentha veridis* extract using the protocol described in the Materials and Methods section. It was immediately analyzed by GC-MS; *m/z* 100 (M^+, 1%), 82 ($[M\text{-}H_2O]^+$, 22), 72 (32), 71 (17), 67 (21), 57 (75), 56 (91), 53 (9), 44 (87), 41 (100), 29 (73), and 27 (74).

3*Z*-Hexenal

3*Z*-Hexenal was prepared from 13*S*-hydroperoxy linolenic acid in the presence of *M. veridis* extract using the protocol described in the Materials and Methods section: *m/z* 98 (M^+, 5%), 97 (3), 83 (14), 80 (8), 69 (33), 55 (28), 42 (20), 41 (100), 39 (36), 29 (19), and 27 (17).

Table 1
Hexanol (nmol) Measured in Coupled-Reaction System (Cleavage/Oxidation) After 15-min Reaction at 15°C[a]

	Substrate (m*M*)			
	1	2	3	4
HPLS alone	8	11.5	9.2	10.3
HPLS + ADH + NAD	10.3	9.2	7	5
HPLS + NAD	4.2	2.2	7	4.2
HPLS + NADP	1.7	1	1.8	1.8

[a]13*S*-Hydroperoxy linoleic acid was used as the substrate in the presence of 1.8 mL of mint extract and phosphate buffer, pH 7.0, in 2 mL total volume stirred at 100 rpm. Horse liver ADH (3 U) was used in some reactions. Cofactors were used at a rate of 5 m*M*.

HPLS, hydroperoxidelyase; ADH, alcoholdehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

2*E*-Hexenal

2*E*-Hexenal is the product of isomerization of the previous compound: *m/z* 98 (M^+, 4%), 97 (5), 83 (32), 80 (7), 69 (32), 57 (27), 55 (52), 42 (50), 41 (99), 39 (100), 29 (79), and 27 (66).

1-Hexanol

1-Hexanol was prepared from the 13*S*-hydroperoxy linoleic acid in the coupled-enzyme system (mint HPLS/horse liver ADH in the presence of NADH): *m/z* 102 (M^+, 0.2%), 85 (6), 84 (7), 73 (4), 69 (65), 56 (97), 55 (100), 53 (9), 43 (82), 41 (81), 39 (57), 31 (82), 29 (84), and 27 (86).

3*Z*-Hexenol

3*Z*-Hexenol was prepared from the 13*S*-hydroperoxy linolenic acid in the coupled-enzyme system (mint HPLS/horse liver ADH in the presence of NADH): *m/z* 100 (M^+, 3%), 82 (43), 72 (4), 69 (27), 67 (100), 65 (6), 57 (17), 55 (56), 53 (16), 43 (16), 41 (99), 39 (38), 31 (30), 29 (17), and 27 (16).

Results and Discussion

Selective Synthesis of Volatile Compounds in Coupled-Enzyme System

Incubation of 13-hydroperoxy linoleic acid at pH 7.0 in the presence of aqueous extract of mint not only produces the corresponding C_6-aldehyde but also a small quantity of alcohol *(19)*. To support the production of only one of the volatile compounds (aldehyde or alcohol), a system of coupling between HPLS and ADH was tested in the presence of different cofactors. An aqueous extract of mint leaves was used as the source of HPLS. ADH from horse liver and ADH from yeast were tested. To reach an exclusive synthesis of aldehyde without alcohol, HPLS and ADH were coupled in the presence of NAD(P) as cofactors, to oxidize the alcohol in aldehyde (Table 1). To make a reaction in which aldehyde would be reduced into alcohol, the NAD(P)H cofactors were used (Table 1).

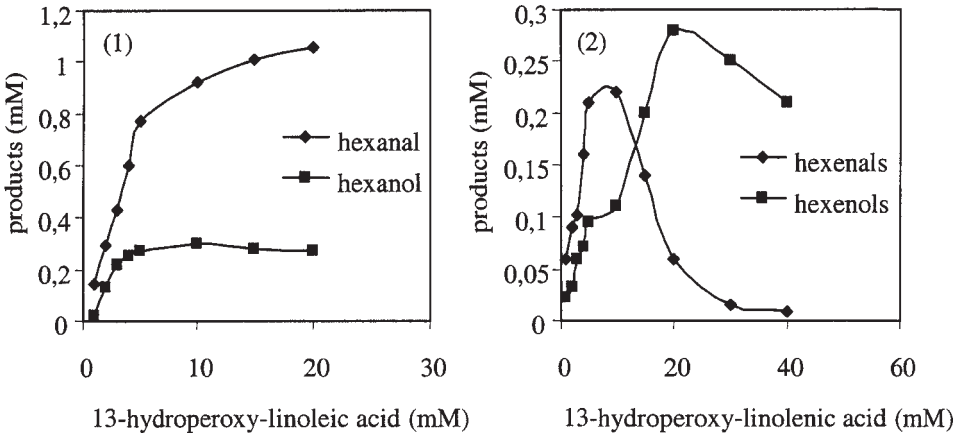

Fig. 2. Aldehyde and alcohol production (μmol) after 15-min reaction in coupled-reaction system (cleavage/oxidation). **(A)** 13-Hydroperoxy linoleic acid or **(B)** 13-hydroperoxy linolenic acid were used as substrates in the presence of 1.8 mL of mint extract, phosphate buffer, pH 7.0; 3 U of horse liver alcoholdehydrogenase; and 5 m*M* cofactor. The medium (2 mL) was stirred at 100 rpm at 15°C. Total hexenal and hexenol concentrations were calculated by considering the sum of peak areas of the corresponding isomers (2*E* and 3*Z*).

The coupled cleavage and oxidation to encourage the selective production of hexanal from 13*S*-hydroperoxy linoleic acid was carried out in the presence of different cofactors. The quantity of hexanol produced in the medium was measured out at the end of the reaction (Table 1).

In spite of the weak concentration of hexanol compared with that of hexanal, the balance was displaced toward the production of aldehyde. The best results were obtained when using NADP as cofactor without the addition of ADH. Hexanol production was then reduced 5 to 10 times when compared to the reaction in the presence of HPLS alone. The presence of ADH in the extract of mint used is confirmed by these results. The enzyme would catalyze the oxidation of alcohols in the presence of NADP in excess. The quantity of hexanal produced does not change in the two-enzyme system.

The two successive reactions of cleavage of 13*S*-hydroperoxy linoleic acid and reduction of aldehyde were also coupled with the objective of producing the corresponding alcohol. The same enzymes were tested but in the presence of reduced cofactors to promote the reduction. The production of alcohols and aldehydes was measured at the end of the reaction by headspace-GC. Cleavage was catalyzed by HPLS present in the mint extract. The addition of only cofactor (5 m*M* NADH or NADPH) did not increase significantly the reduction of hexanal (data not shown). This suggests that ADH was present in weak concentration in the used enzyme extract. The production of hexanol and 3*Z*-hexenol from their respective substrates—13*S*-hydroperoxy linoleic and 13*S*-hydroperoxy linolenic acids—was studied in the two-enzyme system (mint HPLS/HLADH, NADH; Fig. 2). Results found using yeast ADH were comparable with those found using horse liver ADH (Fig. 2).

Table 2
Rate of Disappearance of 13-Hydroperoxy Linoleic Acid (n*M*/min) as Function of Substrate Concentration in 0.2 *M* Phosphate Buffer, pH 6.5, at 25°C, 1-mL Total Volume[a]

Substrate (µ*M*)	Substrate consumption rate (n*M*/min)	
	Cleavage	Cleavage/reduction
60	4	12
100	12	24

[a]Cleavage reaction: in the presence of HPLS (100 µL of extract). Cleavage/reduction reaction, two-enzyme system: hydroperoxide-lyase (100 µL of extract)/3 U of yeast alcoholdehydrogenase, 1.5 m*M* reduced nicotinamide adenine dinucleotide.

Table 3
Rate of Disappearance of 13-Hydroperoxy Linoleic Acid (n*M*/min) as Function of Concentration of ADH or NADH Added to Medium[a]

	Rate (n*M*/min)				
	8	16	20	40	46
ADH (U)	3	0.9	3	3	3
NADH (m*M*)	0.8	1.5	1	1.5	2

[a]The substrate was converted in the two-enzyme system: coupled-HPLS (100 µL of extract)/ADH-NADH in 0.2 *M* phosphate buffer, pH 6.5, at 25°C. The initial substrate concentration was 100 µ*M* in a 1-mL total volume.

ADH, alcoholdehydrogenase; NADH, reduced nicotinamide adenine dinucleotide.

Reduction activity was observed after the cleavage when coupling HPLS to horse liver ADH. However, at low hydroperoxide concentration, this activity does not lead to an important synthesis of hexanol or hexenols as major products from 13*S*-hydroperoxy linoleic or 13*S*-hydroperoxy linolenic acids, respectively. Aldehyde remains the major product. When using a substrate concentration >15 m*M*, alcohol concentration was more important than the aldehyde obtained in the two-enzyme system, reaching 95% of the products.

Conversion Rate in Two-Enzyme System

The evolution of the coupled-enzyme system was followed by spectrophotometric measurement of the decrease in hydroperoxy fatty acid concentration. This initial substrate contains conjugated double bonds that absorb at 234 nm and disappear during the reaction of cleavage. The rate of disappearance of 13*S*-hydroperoxy linoleic acid increased as a function of the initial substrate concentration in the two-enzyme system (Table 2). The reaction rate was higher than that found in the cleavage reaction in the

presence of HPLS (Table 2). Coupling of the enzymatic reactions not only reduced the number of steps but also allowed us to increase the conversion rate. The proximity of ADH and its substrate aldehyde is a catalytic advantage and is favorable to the HPLS-catalyzed cleavage of hydroperoxy fatty acid. When increasing ADH or NADH concentrations, the conversion rate in the two-enzyme system increased (Table 3). These results confirm that the second reaction catalyzed by ADH was able to favor the first one catalyzed by HPLS.

Conclusion

Two enzymes—HPLS and ADH—were coupled in the same system in order to improve the specific synthesis of C_6-aldehyde or alcohol from 13-hydroperoxy fatty acids. On the one hand, when using the two-enzyme system HPLS/ADH, the purity of hexanal was improved, because the cleavage of 13-hydroperoxy linoleic acid was coupled to the oxidization of the small amount of hexanol. On the other hand, coupling of the cleavage to the reduction of the aldehyde did not permit production of alcohol exclusively. A large proportion of the product remained as aldehyde. The presence of the second reaction catalyzed by ADH favors the evolution of the first one catalyzed by HPLS. The total conversion rate improved in the coupled-enzyme system. All the product structures were confirmed by GC-MS analysis.

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References

1 Hatanaka, A., Sekiya, J., and Kajiwara, T. (1978), *Phytochemistry* **17,** 869–872.
2 Sekiya, J., Kajiwara, T., Munechika, K., and Hatanaka, A. (1983), *Phytochemistry* **22,** 1867–1869.
3 Hatanaka, A. (1993), *Phytochemistry* **34,** 1201–1218.
4. Gardner, H. W. (1995), *HortScience* **30,** 197–204.
5. Gargouri, M. (2001), in *Recent Research Development in Oil Chemistry*, vol. 5, Pandalai, S. G., ed., Transworld Research Network, Trivandrum, India, pp. 13–37.
6 Clark, G. S. (1990), *Perfumer Flavorist* **15,** 47–52.
7. Brunerie, P. (1989), French patent 89, 12 901.
8 Fauconnier, M. L., Perez, A. G., Sanz, C., and Marlier, M. (1997), *J. Agric. Food Chem.* **45,** 4232–4236.
9 Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T., and Hatanaka, A. (1991), *Phytochemistry* **30,** 2109–2113.
10 Shibata, Y., Matsui, K., Kajiwara, T., and Hatanaka, A. (1995), *Biochem. Biophys. Res. Commun.* **207,** 438–443.
11 Itoh, A. and Vick, B.A. (1999), *Biochim. Biophys. Acta* **1436,** 531–540.
12 Gardner, H. W. (1991), *Biochim. Biophys. Acta* **1084,** 221–239.

13 Hatanaka, A. (1996), *Food Rev. Int*. **12,** 303–350.
14. Kim, I. S. and Grosch, W. (1981), *J. Agric. Food Chem.* **29,** 1220–1225.
15 Gargouri, M. and Legoy, M. D. (1998), *Biotechnol. Lett.* **20,** 23–26.
16 Schreier, P. and Lorenz, G. (1982), *Z. Naturforsch.* **37c,** 165–173.
17 Hatanaka, A., Kajiwara, T., Sekiya, J., and Inouye, S. (1982), *Phytochemistry* **21,** 13–17.
18. Vick, B. A. and Zimmerman, D. C. (1976), *Plant Physiol.* **57,** 780–788.
19 Gargouri, M., Drouet, P., and Legoy, M. D. (2004), *J. Biotechnol.* **111,** 59–65.
20 Galliard, T. and Phillips, D. R. (1976), *Biochim. Biophys. Acta* **431,** 278–287.
21 Matsui, K., Shibata, Y., Kajiwara, T., and Hatanaka, A. (1989), *Z. Naturforsch.* **44c,** 883–885.
22. Matoba, T., Sakura, A., Taninoki, N., Saitoh, T., Kariya, F., Kuwahata, M., Yukawa, N., Fujino, S., and Hasegawa, K. (1989), *J. Food Sci.* **54,** 1607–1610.
23. Legoy, M. D., Kim, H. S., and Thomas, D. (1985), *Process Biochem*. **20,** 145–148.
24. Lortie, R., Villaume, I., Legoy, M. D., and Thomas, D. (1989), *Biotechnol. Bioeng.* **33,** 229–232.
25. Branden, C. V., Jornvall, H., Eklund, H., and Furugren, B. (1975), in *The Enzymes*, 3rd ed., vol. 11, Boyer, P. D., ed., Academic Press, New York, pp. 103–187.
26. Hatanaka, A., Kajiwara, T., and Harada, T. (1975), *Phytochemistry* **14,** 2589–2592.