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Development of a Biocatalytic Process for the Production of C6-Aldehydes from Vegetable Oils by Soybean Lipoxygenase and Recombinant Hydroperoxide Lyase

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Volatile C6- and C9-aldehydes and alcohols are widely used as food flavors to reconstitute the "fresh green" odor of fruits and vegetables lost during processing. To meet the high demand for natural flavors, an efficient, cheap, and versatile biocatalytic process was developed to produce C6-aldehydes on a large scale. Vegetable oils were converted by soybean lipoxygenase and recombinant hydroperoxide lyase into hexanal and (2*E*)- or (3*Z*)-hexenal. In contrast to plant extracts, generally used as enzyme sources, high molar conversions were obtained with recombinant hydroperoxide lyase (50% for hexanal and 26% for hexenal formation), and no side products were formed. Furthermore, recombinant hydroperoxide lyase lacks isomerase activity, allowing production of (3*Z*)-hexenal, which could not be obtained in previously described processes. Recombinant hydroperoxide lyase is stable and can be stored at 4 °C for 1 month without significant loss of activity.

KEYWORDS: Lipoxygenase; hydroperoxide lyase; C6-aldehydes; biocatalysis; flavors

INTRODUCTION

Volatile C6- and C9-aldehydes and alcohols, such as hexanal, (3*Z*)- and (2*E*)-hexenal, (3*Z*)- and (2*E*)-nonenal, and (3*Z*,6*Z*)- and (2*E*,6*Z*)-nonadienal, are the main contributors to the characteristic odor of plants. They are widely used as food flavors to reconstitute the "fresh green" odor of fruits and vegetables, lost during processing. These compounds can be extracted from plants or synthesized. Chemical synthesis is not favored because consumers have a strong preference for natural food additives. Extraction is very expensive because of the low abundance of these short-chain aldehydes and alcohols in plants. Therefore, development of a biocatalytic process is required to produce these compounds on a large scale.

In plants, C6- and C9-aldehydes and alcohols are produced by the lipoxygenase pathway (**Figure 1**) and are involved in wound healing and pest resistance (*1*, *2*). Linoleic (OD) and α-linolenic acids (OT) are dioxygenated by lipoxygenase (LOX) to form 13- or 9-hydroperoxy-linole(n)ic acids (HPOD/T). The 13-hydroperoxy fatty acids can subsequently be cleaved by hydroperoxide lyase (HPL) into 12-oxo-(9*Z*)-dodecenoic acid and hexanal or (3*Z*)-hexenal, whereas the 9-hydroperoxy fatty acids can be cleaved into 9-oxononanoic acid and (3*Z*)-nonenal or (3*Z*,6*E*)-nonadienal. The (3Z)-aldehydes easily isomerize to their (2*E*)-enal isomers and can be reduced to their corresponding alcohols by alcohol dehydrogenase.

Until now, plant extracts or cell cultures are the most common enzyme sources for biocatalytic production of C6- and C9-aldehydes and alcohols. Large-scale conversion of fatty acids by lipoxygenase has successfully been accomplished (*3*−*6*). The subsequent conversion of hydroperoxy fatty acids by hydroperoxide lyase, however, caused problems. HPL in plant extracts is very unstable, and molar conversion rates were often <1% (*7*−*10*). Screening of different vegetables resulted in yields of 36% for hexanal and 24% for (2*E*)-hexenal (*11*). With hydroperoxide lyase from mung bean seedlings and linseed oil, molar conversions of 39% for (2*E*)-hexenal and 21% for hexanal could be obtained (*12*). However, no (3*Z*)-hexenal was formed due to rapid isomerization to (2*E*)-hexenal. Furthermore, the crude extracts contained other enzymes that used hydroperoxy fatty acids as substrates, such as allene oxide synthase and peroxygenase, leading to formation of undesired side products.

Recombinant expression would be an excellent method to increase the availability of hydroperoxide lyase for a biocatalytic process. The expression system we described before (*13*) has made it possible to obtain large quantities of stable hydroperoxide lyase with a high specificity for 13-hydroperoxylinoleic and α-linolenic acids. Furthermore, the recombinant HPL forms (3*Z*)-hexenal, a compound that has been very difficult to produce because of its instability. Here, we describe the optimization of this expression system. The optimal reaction conditions of the

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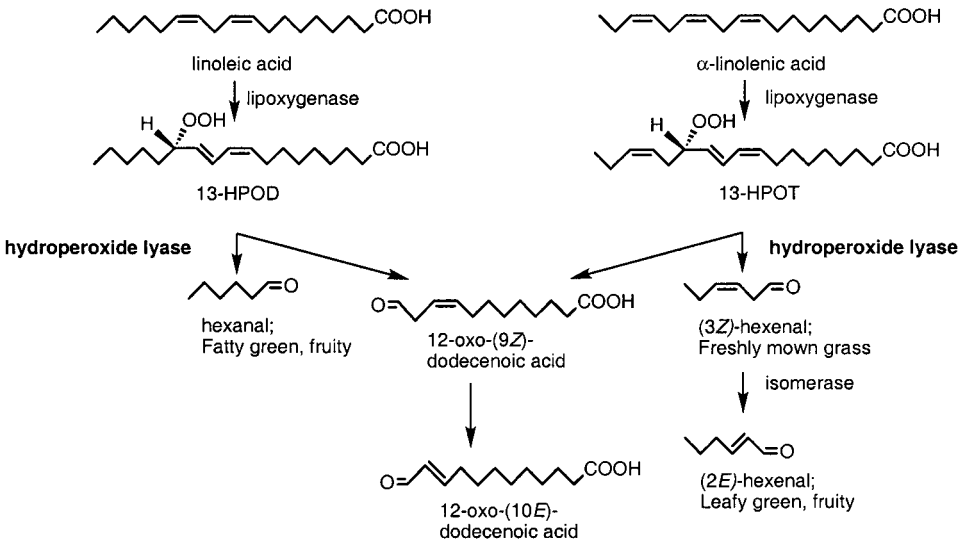

Figure 1. C6-Aldehyde-forming system in plants. C9-Aldehydes are formed similarly from 9-hydroperoxy fatty acids.

recombinant HPL were determined, as well as the kinetic parameters. A versatile biocatalytic process has been developed in which vegetable oils are converted by soybean lipoxygenase and recombinant hydroperoxide lyase to hexanal and (3*Z*)- or (2*E*)-hexenal.

MATERIALS AND METHODS

Expression and Isolation of HPL. Alfalfa HPL was expressed in M15 *Escherichia coli* cells containing the pQE32 vector (Qiagen) with the *CYP74B4v1* gene without N-terminal sequence coding for the first 22 amino acids [EMBL Database, accession no. AJ249245 (*13*)]. For optimal expression, cells were grown at 37 °C with maximal stirring and O_2 flow, in 1.5× LB medium supplemented with 0.5% glucose, 1× minimal medium buffer, 25 μg mL^{-1} kanamycin, and 50 μg mL^{-1} ampicillin. Expression of HPL was induced at an A_{600} of 0.7 by the addition of 1 mM isopropyl β-D-thiogalactoside (IPTG), and the cells were further grown for 22 h at 25 °C to prevent sequestering of HPL in inclusion bodies. Cells were harvested by centrifugation, and the supernatant was discarded. The cell pellets were resuspended in 50 mM potassium phosphate buffer, pH 8, and sonicated on ice. Triton X-100 from a 10% solution (w/v) was added to a final concentration of 0.5% after sonication, and the suspension was recentrifuged. HPL was present in the supernatant. All steps were carried out at 4 °C. For the determination of the initial turnover rates, K_m and V_{max}, HPL was purified by immobilized metal affinity chromatography as described (*13*).

HPL Activity Measurements. HPL activity was determined in 50 mM potassium phosphate buffer, pH 8.25, containing 100 μM substrate, at 25 °C. The decrease of A_{234} due to cleavage of the substrate was followed spectrophotometrically. One unit of activity (U) corresponds to the amount of enzyme that converts 1 μmol of substrate per minute. The maximal turnover number was determined by measuring the substrate conversion at various enzyme/substrate ratios. Apparent K_m and V_{max} values were determined from the means of six determinations with concentrations of substrate ranging from 5 to 150 μM. The data were fitted to the standard Michaelis−Menten kinetic equation (Graphpad Prism).

Biocatalytic Processes. Hydrolyzed safflower oil (73% linoleic acid) was used as linoleic acid source, and hydrolyzed linseed oil (44% linolenic acid/20% linoleic acid) was used as linolenic acid source. The reactions were performed in a 7 or 2 L stirred tank bioreactor (2 or 0.5 L working volumes, respectively), equipped with four baffles and two impellers (Applikon Dependable Instruments, ADI 1035, Schiedam, The Netherlands). The bioreactor was equipped with a thermometer, an oxygen electrode, and a pH electrode and was controlled by an Applikon Bio Controller ADI 1030. The reaction mixture contained 10 g L^{-1} defatted soybean flour as lipoxygenase source in 50 mM potassium phosphate buffer, pH 8.25, at 20 °C. The pH was automatically maintained by the addition of 3 M NaOH. Safflower or linseed oil was linearly added over 1 h to a final concentration of 40 mM linole(n)ic acid. Oxygen saturation was kept at 100% by the addition of pure oxygen or air. Stirring speed was 400 rpm. When the lipoxygenase reaction was finished and A_{234} did not increase anymore, the oxygen flow was stopped and 115 U of HPL/mmol of formed 13-hydroperoxylinoleic acid or 400 U/mmol of 13-hydroperoxylinolenic acid was added. The HPL reaction was performed in a closed vessel with a small headspace volume at 10 °C with slow stirring. If the hydroperoxy fatty acids were extracted from the LOX mixture prior to incubation with HPL, the LOX mixture was acidified with HCl to pH 4. The extraction was performed twice with diethyl ether, and the combined ether fractions were frozen to remove water and concentrated by evaporation. In the combined LOX/HPL system, HPL was added to the lipoxygenase reaction mixture in a ratio of 115 U/mmol of linoleic acid added.

Product Analysis. The amount of hydroperoxy fatty acids was determined by HPLC (Cosmosil 5C18-AR column, Waters; MeOH/H_2O/THF/HAc, 35/35/30/0.1, 1 mL min^{-1}) with detection at A_{234}. To quantify the amount of volatiles formed, samples were diluted 1:1 with 2-propanol containing 20 mM octanal as internal standard and analyzed by GC-FID (HP-Innowax column, 30 m × 0.32 mm, 0.25 μm film thickness; temperature gradient, 40−200 °C, 10 °C min^{-1}; injection temperature, 200 °C). Nonvolatile products were extracted from the reaction mixture, reduced, methylated, trimethylsilylated, and analyzed by GC-MS as described before (*13*).

RESULTS

Optimization of HPL Expression and Isolation Conditions. The influences of expression temperature and medium on the production of active HPL in *E. coli* were determined. The optimal expression temperature appeared to be 25 °C (**Figure 2**). At higher temperatures most of the expressed HPL was inactive and probably located in inclusion bodies. Enriched medium resulted in a higher cell density and an increased amount of HPL produced. HPL expression was maximal in the late log-phase and stationary phase. As cells start to die after 24 h, longer expression times will not lead to a higher expression level. For optimal expression 1 mM IPTG had to be added at $A_{600} < 0.8$ (**Figure 3A**). However, also with lower concentrations of IPTG (until 100 μM) a reasonably good expression was obtained. Because IPTG is rather expensive, the optimal amount of IPTG for industrial application may be <1 mM, from an economic point of view.

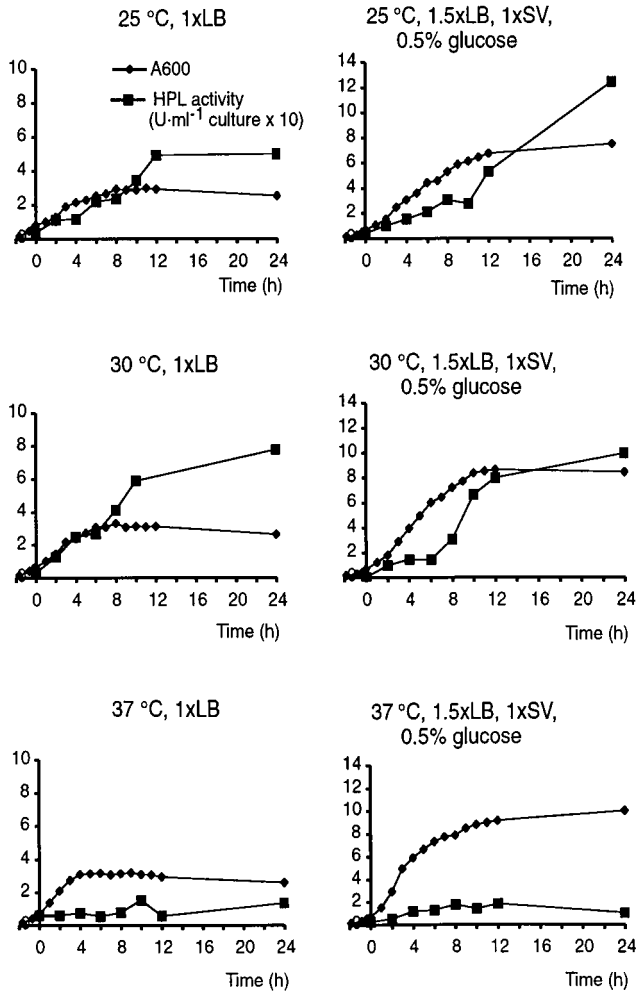

Figure 2. Influence of medium and expression temperature on the amount of active HPL produced. Small-scale *E. coli* cultures expressing HPL were grown at different temperatures in 1× LB medium or in enriched medium (1.5× LB supplemented with 0.5% glucose and 1× minimal medium buffer). Induction occurred by addition of 1 mM IPTG at $A_{600} = 0.7$ ($t = 0$). HPL activity was determined with 13-HPOT as substrate.

The pH of the extraction buffer and percentage of Triton X-100 appeared to have a great influence on the amount of HPL that was solubilized (**Figure 3B**). pH 8 was optimal for extraction, and the optimal amount of Triton X-100 for solubilization was 0.5% (w/v). A second extraction of the cell pellet resulted only in 15% higher yields of HPL. Higher percentages of Triton X-100 may lead to a slightly higher yield but will also result in foaming during the enzymatic reaction and are therefore undesirable.

Expression in a 10 L *E. coli* culture under optimal conditions gave a yield of 3×10^3 or 8×10^3 U of HPL L^{-1} *of E. coli* culture, with 13-HPOD or 13-HPOT as substrate, respectively. The enzyme preparation could be stored at 4 °C for at least 1 month without significant loss of activity.

Optimization of Reaction Conditions. The pH optimum of the HPL reaction with 100 μM substrate appeared to be pH 8.25. The other HPL isoenzymes [CYP74B4v2 and v3 (*13*)] showed the same optimal pH. This is remarkable, because the pH optimum of HPL, isolated from alfalfa seedlings, was pH 5.5 (*14*). Although the V_{max} values of recombinant HPL at pH 6 and 8.25 were in the same range (650 U mg^{-1} for 13-HPOT), the K_m at pH 8.25 was much lower than at pH 6 (42 μM at pH 8.25 instead of 140 μM at pH 6 for 13-HPOT). An increase of

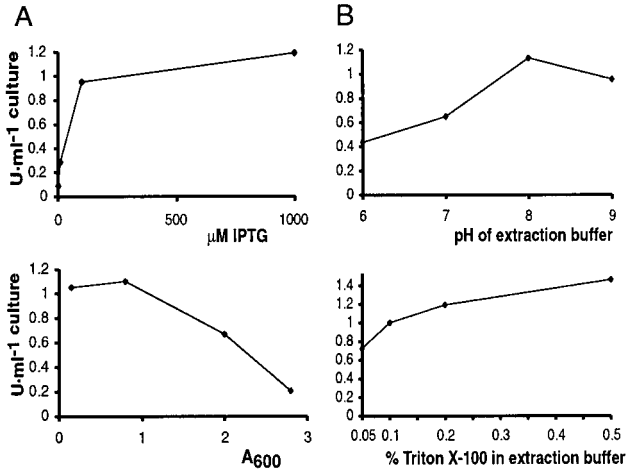

Figure 3. (A) Influence of the amount of IPTG and cell density at the time of induction on the expression of HPL; (B) influence of pH of the extraction buffer and Triton X-100 concentration on the solubilization of HPL. Small-scale *E. coli* cultures expressing HPL were grown at 25 °C in 1× LB medium. HPL activity was determined with 13-HPOT as substrate.

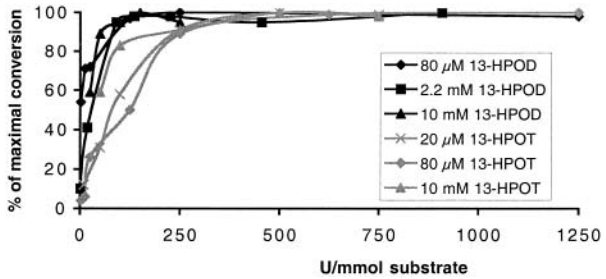

Figure 4. Maximal turnover of substrate by HPL. Different amounts of HPL were incubated with various substrate concentrations. The maximal conversion obtained at each substrate concentration was set to 100%.

reaction temperature from 10 to 50 °C led to an increase in initial reaction velocity. However, at temperatures >20 °C, HPL was rapidly inactivated, which led to a lower conversion.

The initial turnover rates with 100 μM 13-HPOD or 13-HPOT were 330 or 750 s^{-1}, respectively. To convert 1 μmol of 13-HPOD or 1 μmol of 13-HPOT, at least 125 or 400 mU of HPL is needed, respectively (**Figure 4**). These maximal turnover numbers of recombinant HPL are equal to the turnover numbers of HPL in a crude extract of alfalfa. From these numbers it could be derived that an enzyme molecule can convert $\sim 1.6 \times 10^5$ molecules of 13-HPOD or 0.9×10^5 molecules of 13-HPOT before it is inactivated. Inactivation of HPL was independent of the substrate or product concentration and of the presence of glycerol, Triton X-100, or *E. coli* membrane fragments. This indicates that inactivation is caused by reaction intermediates. Suicide inactivation was also suggested by Matsui et al., who observed destruction of the heme group during the reaction (*15*).

Development of a Biocatalytic Process. A process in which the LOX and HPL reactions take place simultaneously has the advantage that there is no product inhibition of LOX, because all product formed by LOX is immediately converted by HPL. As HPL was not inhibited by soybean flour or safflower oil, such a process was worthwhile to study. Although 80% of the linoleic acid added was converted in this one-step process, the hexanal yield appeared to be quite low (17%) (**Figure 5**). Besides low amounts of 9-hydroperoxylinoleic acid and the coproducts 12-oxo-(10*E*)-dodecenoic acid and 12-oxo-(9*Z*)-dodecenoic acid, no other products were detected, due to the high specificity of recombinant HPL. The addition of oxygen

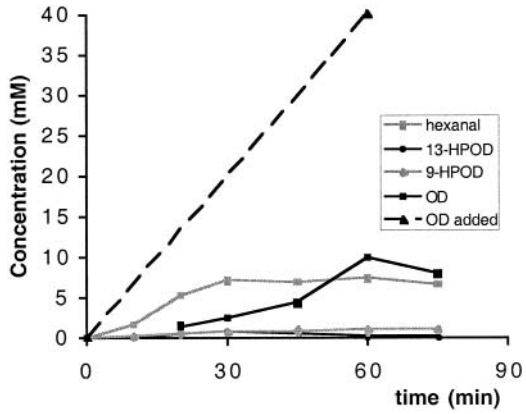

Figure 5. Hexanal formation in a one-step LOX-HPL process. Hydrolyzed safflower oil was linearly added over 1 h to a concentration of 40 mM OD. HPL was simultaneously added in a ratio of 115 U/mmol of OD. The reaction mixture contained 10 g L^{-1} soybean flour.

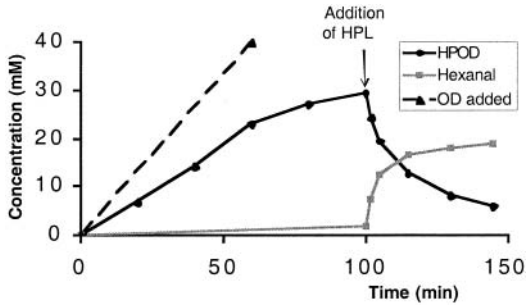

Figure 6. Hexanal formation in a two-step biocatalytic process. Hydrolyzed safflower oil was linearly added over 1 h to a concentration of 40 mM OD. HPL was added in a ratio of 115 U/mmol of formed13-HPOD after the LOX reaction was finished and the oxygen flow was stopped.

or air needed for the LOX reaction probably led to evaporation of the volatile aldehydes formed in the HPL reaction. Therefore, a two-step process in which the oxygen flow was stopped before HPL addition was preferred. The yield of the lipoxygenase reaction was again typically ~80%. Subsequently, up to 65% of the formed 13-hydroperoxylinoleic acids was converted into hexanal, indicating that evaporation was indeed one of the reasons for the low yield of the combined process (**Figure 6**). The overall yield of the two-step process was 50%. Another cause of product loss might be the binding of aldehydes or hydroperoxy fatty acids to proteins in the reaction mixture (*16*, *17*). To reduce the protein concentration during the HPL reaction, the hydroperoxy fatty acids were extracted from the LOX mixture prior to addition of HPL. The hexanal yield appeared to be independent of protein and substrate concentrations (ranging from 0.1 to 25 mM 13-HPOD), and extraction of hydroperoxylinoleic acid did not lead to higher yields.

Conversion of linseed oil by soybean lipoxygenase gave yields of ~60%. When the LOX mixture was incubated with HPL, (3*Z*)-hexenal was formed, which slowly isomerized to (2*E*)-hexenal (**Figure 7**). The isomerization velocity increased with increasing temperature. Because linseed oil contains both linoleic and linolenic acid, hexanal was formed as well. Due to the preference of HPL for 13-HPOT, hexanal formation was much slower than hexenal formation. If the reaction was stopped after 2 min, a high percentage of (3*Z*)-hexenal could be obtained (~80% of the C6-aldehydes). Although 85% of 13-HPOT was converted after 2 min, the hexenal yield was only 43%. The appearance in time of a strong brown-yellow color indicated the formation of Schiff bases with proteins *(18)*. Extraction of

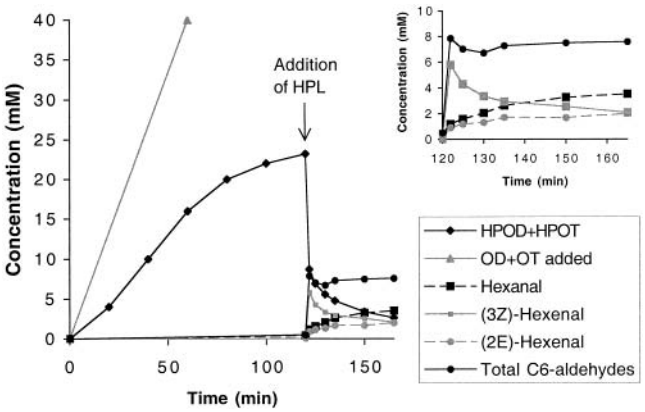

Figure 7. C6-Aldehyde formation in a two-step biocatalytic process with hydrolyzed linseed oil as substrate (linearly added over 1 h to a concentration of 40 mM OD + OT). HPL was added in ratios of 115 U/mmol of formed 13-HPOD and 400 U/mmol of formed 13-HPOT after the LOX reaction was finished and the oxygen flow was stopped. (Inset) Enlargement of C6-aldehyde formation after addition of HPL.

Table 1. Starting Material Needed per Gram of Produced Aldehyde

	hexanal	(3*Z*)- or (2*E*)-hexenal
safflower or linseed oil	8.2 g	26 g
K_2HPO_4	5.1 g	15 g
KH_2PO_4	0.3 g	0.9 g
soybean flour	5 g	15 g
E. coli culture expressing HPL	0.6 L	1.6 L
molar conversion	50%	26%

the hydroperoxy fatty acids from the LOX mixture prior to incubation with HPL did not lead to a higher hexenal yield after 2 min. The yield could be increased only by lowering the substrate concentration.

For the isolation of the formed aldehydes from the reaction mixture, the following methods were compared: (1) extraction with organic solvents, (2) steam distillation, and (3) binding to MN-100 (Purolite), a resin that is often used for flavors. Extraction with diethyl ether or ethyl acetate gave very high yields and high purity, because a large fraction of the fatty acids remained in the water phase at the used pH of 8.25. Separation of the organic layer from the water phase, however, was difficult because of denatured proteins on the interphase. Steam distillation also gave good yields but had to be performed under anaerobic conditions to prevent oxidation of the aldehydes to acids. Furthermore, this method was not suitable for (3*Z*)-hexenal because the high temperature led to isomerization to (2*E*)-hexenal. Binding to MN-100 and elution with organic solvent gave low yields (~50%), because the presence of proteins in the mixture led to strongly decreased binding of the aldehydes to the resin.

DISCUSSION

The biocatalytic process described here allows rapid, efficient, and cost-effective generation of C6-aldehydes, including (3*Z*)-hexenal. The dioxygenation of linoleic and linolenic acids from hydrolyzed vegetable oils by soybean LOX results in high yields of hydroperoxy fatty acids, as described before (*3*−*6*). However, in contrast to previously described processes with plant extracts or cell cultures, the use of recombinant HPL leads to higher aldehyde yields and fewer side products. The total amounts of starting material for the production of 1 g of hexanal or (3*Z*)- or (2*E*)-hexenal are shown in **Table 1**. The HPL reaction can

easily be scaled up as it is a very simple batch process; no extensive mixing is required, and no foaming occurs.

The process is versatile, as a range of products can be obtained by changing the reaction conditions. Pure hexanal can be produced from safflower oil, and the absence of isomerase activity in recombinant HPL makes it possible to produce (3*Z*)-hexenal from linseed oil. Short reaction times result in a high percentage of (3*Z*)-hexenal, whereas longer incubations give a mixture of hexanal, (3*Z*)-hexenal, and (2*E*)-hexenal. (3Z)-Hexenal can rapidly be isomerized to (2*E*)-hexenal by heating. Using commercial baker's yeast (*Saccharomyces cerevisiae*), the formed aldehydes can easily be reduced to the corresponding alcohols (*11*, *19*).

Although >90% of the hydroperoxy fatty acids were converted by HPL, the C6-aldehyde yield was significantly lower. Hexenal seemed to form Schiff bases with proteins, as a strong yellow-brown color appeared in time. Extraction of the hydroperoxy fatty acids from the LOX mixture prior to incubation with HPL did not lead to higher yields, indicating that not only soybean proteins but also *E. coli* proteins from the HPL mixture are causing product loss. The protein concentration can be minimized by using purified HPL. This is, however, an expensive and time-consuming technique, making it less suitable for industrial purposes (*13*). Hexanal did not bind to proteins to a large extent, as the hexanal yield was independent of the substrate and protein concentrations. Products formed from C6-aldehydes in secondary reactions could not be determined. Polymerization of hydroperoxy fatty acids might be another reason for product loss (*20*).

ABBREVIATIONS USED

HPL, hydroperoxide lyase; 13-HPOD, 13-hydroperoxylinoleic acid; 13-HPOT, 13-hydroperoxylinolenic acid; IPTG, isopropyl β-D-thiogalactoside; LOX, lipoxygenase; OD, linoleic acid, (9*Z*,-12*Z*)-octadecadienoic acid; OT, α-linolenic acid, (9*Z*,12*Z*,15*Z*)-octadecatrienoic acid.

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