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Purification of Hydroperoxide Lyase from Green Bell Pepper (*Capsicum annuum* L.) Fruits for the Generation of C6-aldehydes in Vitro

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The aim of this work was to compare the efficiency of different extracts of hydroperoxide lyase from green bell peppers in producing aldehydes: a crude extract, a chloroplastic fraction, and a purified enzyme were investigated. From a crude extract, the HPO lyase was purified by ion-exchange chromatography with a 22.3-fold increase in purification factor. Analysis by SDS−PAGE electrophoresis under denaturating conditions showed only one protein with a molecular weight of 55 kDa, whereas size-exclusion chromatography indicated a molecular weight of 170 kDa. A maximum of 7500 mg of aldehydes per g of protein was obtained with the purified enzyme within 20 min of bioconversion compared to 392 and 88 mg of aldehydes per g of protein within 50 and 60 min, respectively, for the chloroplast fraction and the crude extract.

KEYWORDS: Fatty acid hydroperoxide lyase; green bell pepper; *Capsicum annuum* L.; chloroplasts; purification; volatile aldehydes

INTRODUCTION

Formation of C6 volatile aldehydes (hexanal, *cis*-3-hexenal, and *trans*-2-hexenal) and their corresponding alcohols (hexanol, *cis*-3-hexenol, and *trans*-2-hexenol) via the lipoxygenase pathway is largely responsible for the fresh green odor of many vegetables and fruits (*1*, *2*).

In plants, the alcohols are generated via three sequential enzymatic reactions: lipoxygenase (LOX) transforms polyunsaturated fatty acids (linoleic and linolenic acids) into hydroperoxides, which are cleaved by hydroperoxide lyase (HPO lyase) into aldehydes and oxo-acids. These aldehydes can be further reduced by an alcohol dehydrogenase (ADH) into alcohols (*3*). In the majority of plants, HPO lyase is specific for the 9- or 13-hydroperoxide isomer of linoleic and linolenic acids (*4*) and catalyzes the cleavage of these hydroperoxides into aldehydes and the 9- or 12- carbon oxo-acids, respectively.

To date, purifications of HPO lyase have been reported from plant sources including soybean seedlings (*5*), tea leaves (*6*), bell pepper fruits (*7*), tomato leaf (*8*), sunflower hypocotyls (*9*), olive fruits (*10*), cucumbers (*11*), pea seeds (*12*), and tomato fruits (*13*). Recently, HPLs from several plant species have been cloned (*14*−*17*) and found to be heme enzymes grouped in a novel cytochrome P450 family, CYP74B (*7*, *18*). However, these HPO lyases do not need a cofactor such as molecular oxygen or reducing equivalents essential for most P450 enzymes.

HPO lyases have been localized in the envelope membranes of chloroplasts (*2*, *19*, *20*). HPO lyases from green bell pepper have been well characterized and also identified as cytochrome P-450 proteins. Furthermore, the gene encoding HPL from pepper fruit has been cloned and sequenced (*18*).

During the past few years, there has been an increase in the interest for natural products. "Green note" aldehydes and alcools are high-value molecules widely used in the aroma industry. The literature reports several processes based on the use of plant homogenate to produce natural hexenals and hexenols from either linolenic acid or 13-HPOT as substrates (*3*, *21*−*26*). Cell cultures of several fruits were used to produce *cis*-3, *trans*-2-hexenal (*24*, *25*), but the yield of recovery was low (<0.18 mg/kg). A screening of many vegetables and fruits was performed (*26*) where a maximum of 370 mg of hexenal/kg of reaction medium was obtained with a homogenate of green bell pepper and linolenic acid as substrate; however, the conversion rate was low (<16%). Several patents relate processes for providing green note compounds using unsaturated fatty acids, plant extracts as LOX and HPO lyase source, and yeasts as ADH source. A maximum of 1677 μg of *cis*-3-hexenol/g of watermelon foliage was obtained (*27*). An optimized production of *cis*-3-hexenol using radish leaves and *S. cerevisiae* was proposed (*28*), providing 550 mg of *cis*-3-hexenol/kg of leaves. Using freshly prepared puree of whole guava fruits (*Psidium guajava*), 74 mg of hexanol and 10.55 μg of *cis*-3-hexenol/g of guava were produced (*29*). Expression systems for recombinant guava 13-HPO lyase for the production of green notes were also proposed (*30*, *31*); the amount of hexanal formed by the recombinant HPL was 14 g/L of *E. coli* cell lysate (*30*).

In the present work, different preparations of immature green bell pepper HPO lyases were used to produce "green notes"

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aldehydes using the 13-hydroperoxides of linoleic and linolenic acids as substrates. The efficiency of a crude extract, a chloroplast fraction, and a purified enzyme for the production of these aldehydes in sufficient amounts was compared.

MATERIALS AND METHODS

Plant Material. Green bell pepper fruits (*Capsicum annuum* L.) were purchased from a local supermarket. The different varieties were not known.

Chemicals. Olein (Nouracid LE 80), a source of 48% α-linolenic acid and 18% linoleic acid was purchased from Akzo Nobel (France). Isoamyl acetate, hexanal, hexanol, *trans*-2-hexenal, *cis*-3-hexenol, Tween 20, Triton X-100, Triton X-100R, and poly(vinylpyrrolidone) K-30 were purchased from Sigma Chemical Co. (France).

Enzyme Extraction. *Crude Extract.* All procedures were performed at 4 °C. Seeds were removed from immature green bell pepper fruits, and the remaining pericarp (150 g) was cut into small pieces and homogenized with a high-speed homogenizer (Ultraturrax; Janke and Kunkel, Staufen i. Br., Germany) (12,000 rpm; 3 × 1 min) in a 150-mL volume of Tris−HCl buffer (0.1 M, pH 8.5) containing 0.5% (w/v) poly(vinylpyrrolidone) K-30. The homogenate was filtered through a sieve, and the filtrate was used as a crude extract.

Partial HPO Lyase Purification. The crude extract was centrifuged at 30000*g* for 20 min. The pellet was suspended in a 12-mL volume of Tris−HCl buffer (0.1 M, pH 8.5) and centrifuged at 30000*g* for 20 min. The pellet was resuspended in a 14-mL volume of Tris−HCl buffer (20 mM, pH 8.5) containing 0.5% Triton X-100 (w/v). The suspension was stirred for 60 min on ice, then homogenized using a Potter Elvejhem and centrifuged at 30000*g* for 20 min. The solubilized enzyme fraction was filtered through Whatman paper (2.7 μm) and then a 0.45-μm filter (H. V. L. P. filter, Durapore membrane, Millipore, Ireland). A 10-mL volume of this fraction was applied to a DEAE column (Biorad, France) (15 mm i.d. × 70 mm) equilibrated with Tris−HCl buffer (20 mM, pH 8.5) (buffer A). The HPO lyase fraction was eluted with Tris−HCl buffer (20 mM, pH 8.5) containing 1 M KCl (buffer B). The gradient was as follows: 0 to 15% buffer B within 15 min, 15 to 50% buffer B within 120 min, 50 to 100% buffer B within 30 min, and 100% buffer B for 120 min (280 nm wavelength, Econo UV monitor, Biorad, France). Active fractions were concentrated under vacuum on 10 kDa pore-size membranes (30 mL centrifugation tubes, Filtron, France). The concentrated active fraction was then applied to a Superdex G-200 column (Pharmacia, France) (15 mm i.d. × 1000 mm) equilibrated with Tris−HCl buffer (20 mM, pH 8.5) containing 0.5% Triton X-100R (w/v). Proteins were eluted with the same buffer. The major fractions were pooled as described above and assayed for HPO lyase activity.

SDS−Polyacrylamide gel electrophoresis was performed on a 10% (w/v) polyacrylamide gel under denaturating conditions, and protein were stained with Coomassie Brilliant Blue R250 (*32*). Low-molecular-weight markers (Sigma, France) were used as standards: albumin, bovine serum, 66 kDa; ovalbumin, chicken egg, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle, 36 kDa; carbonic anhydrase, bovine erythrocytes, 29 kDa; trypsinogen, bovine pancreas, 24 kDa; trypsin inhibitor, soybean, 20.1 kDa.

Chloroplasts Isolation. All procedures were carried out at 4 °C. A 150-g portion of green bell pepper was homogenized in a mixer (Waring Blender, Krups, Germany) for 1 min in 1 vol Tris−HCl buffer (0.1 M, pH 8.5) containing 0.4 M sucrose. The homogenate was filtered through a sieve. The filtrate was centrifuged at 2500*g* for 2 min. The supernatant was discarded, and the precipitate was washed with buffer and centrifuged at 120*g* for 45 s. The precipitate was discarded, and the supernatant was centrifuged at 2500*g* for 2 min. The supernatant was discarded, and the precipitate was used as the chloroplast fraction.

13-HPO Preparation. The substrate olein (Nouracid LE 80) (31% (w/v)) was suspended in oxygenated borate buffer (0.1 M, pH 9.5) containing 1% (v/v) Tween 80, and soybean flour (21% (w/v)) was used as the lipoxygenase source. The reaction was carried out at 25 °C for 1 h under a constant flow of oxygen and a regulated pH at 9.5. The bioconversion rate was 83% and the best substrate/enzyme ratio was 0.78. Tween 80 was necessary to obtain high values of hydroperoxides. The 13-hydroperoxides of linolenic and linoleic acids (13-HPOT and 13-HPOD) were extracted with diethyl ether, evaporated under vacuum, and kept at −20 °C. The hydroperoxide concentration was determined to be 25,000 μg of active oxygen per g of fatty acid by the peroxide index.

Table 1. Purification of HPO Lyase from Green Bell Pepper Fruits

	total protein (mg)	total activity (μg/min)	specific activity (U/mg prot)[a]	yield (%)	purification (-fold)
crude homogenate (before filtration)	213.22	1232.41	5.78	100.00	1
crude homogenate (after filtration)	17.60	870.12	49.43	70.60	8.55
DEAE					
F1	6.04	779.16	129	63.22	22.31
F2	2.69				
gel filtration					
A[b]	0.99				
B	2.18	62.08	28.48	5.04	4.93
C	1.80	40.62	22.57	3.29	3.90

[a] One unit of HPL activity was defined as the amount of enzyme forming 1 μg of *trans*-2-hexenal per minute. [b] Fractions A, B, and C are issued from fraction Fl.

Enzymatic Assays. HPL activities were assessed by aldehyde analysis using gas chromatography. All bioconversion assays were performed for 1 h at 25 °C and under agitation. The reaction mixture consisted of 4 mL of borate buffer (50 mM, pH 8.5), 1 mL of crude homogenate, 1 mL of purified enzyme or 1 g of chloroplasts (wet weight), and 10 mM of 13-HPOT. One unit of HPO lyase activity was defined as the amount of enzyme producing 1 μg of *trans*-2-hexenal per minute. The volatile aldehydes were extracted with hexane (1:1, v/v) containing isoamyl acetate as an internal standard. The molecules were analyzed on a Hewlett-Packard GC 6890 chromatograph equipped with a flame ionization detector and a HP−Innowax polyethylene glycol capillary column (30 m × 0.32 mm, 0.25 μm thickness). The operational conditions were as follows: H_2, 30 mL/min; air, 300 mL/min; N_2, 3.5 mL/min; detector temperature, 300 °C. The column temperature was programmed from 60 °C to 240 °C at 10 °C/min.

Protein Determination. The protein concentration was measured according to the Lowry method (*33*). Bovine serum albumin (Sigma Chemical, St. Louis, MO) was used as a standard for calibration.

RESULTS AND DISCUSSION

Purification of HPL. Table 1 indicates that from a crude extract of green bell pepper fruits containing 213.22 mg of protein, only 17.60 mg of proteins were recovered after both centrifugation and filtration. Centrifugation of the crude extract was used to obtain a pellet containing membrane-bound HPO lyase that was further solubilized using the non ionic detergent Triton X-100 and subsequently recovered by recentrifugation, in the supernatant fraction.

This protein fraction containing HPO lyase activity was then applied onto a DEAE column where two main protein peaks were obtained (**Figure 1**). Fraction F1 was eluted using 200−300 mM KCl in Tris−HCl buffer (20 mM, pH 8.5). The HPO lyase specific activity of fraction F1 was 129 U/mg protein with a purification factor of 22.31-fold and recovery of 63.22%. Fraction F2 contained 2.69 mg protein but no HPL activity. Fraction F1 was subjected to a Superdex G200 column chromatography, and proteins were eluted with Tris−HCL buffer (20 mM, pH 8.5) containing 0.1% Triton X-100R. The presence of Triton X-100R proved to be essential for the elution of the HPL protein, which is similar to that reported for HPL obtained from ripening strawberry fruits (*34*), tomato leaf (*8*), and green bell pepper fruits (*7*). Fraction F1 was subsequently separated into three fractions: A, B, and C (**Figure 2**), where fraction A contained 0.99 mg of protein and no HPL activity,

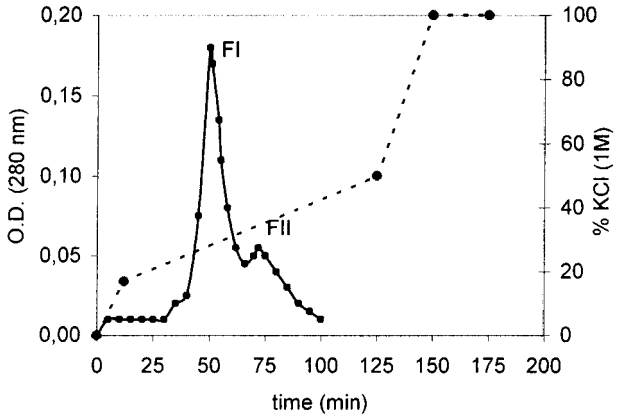

Figure 1. Ion-exchange chromatography of green bell pepper HPO lyase: protein measured at 280 nm (–■–), KCl gradient (0–1 M) (- - ● - -).

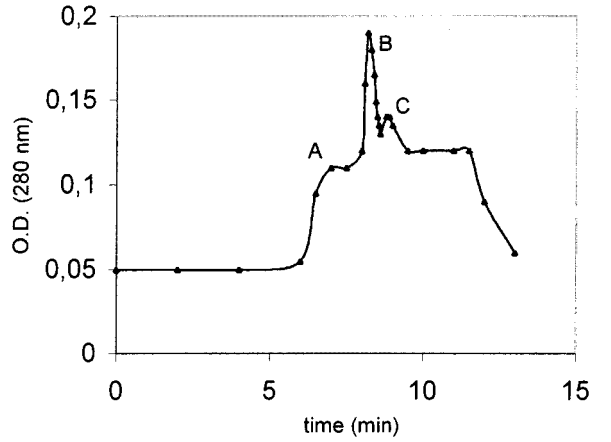

Figure 2. Size-exclusion chromatography of fraction F1 obtained by DEAE-exchange chromatography; protein measured at 280 nm.

while fractions B and C contained 2.18 and 1.80 mg of protein, respectively, and had corresponding specific activities of 28.48 and 22.57 U/mg proteins. Fractions B and C also indicated similar purifications of 4.93-fold and 3.90-fold and recoveries of 5.04 and 3.29%, respectively (**Table 1**). A small loss of total activity after gel filtration was probably due to protein denaturation during this last step of purification. Several compounds such as dithiothreitol (5 mM), α-tocopherol (0.2 mM), nordihydroguaiaretic acid (0.2 mM), or butylated hydroxyanisole (0.5 mM) have a protector effect on HPL activity (*35*). However, none of these compounds were added to the purification process.

The presence of many bands in the crude homogenate but only one major protein band of 55 kDa relative molecular weight in fractions FI and B were detected by SDS−PAGE analysis. Fraction C also showed one major band of 55 kDa and a few minor ones having less than 29 kDa relative molecular weight (data not shown). This protein of 55 kDa could therefore be a monomer of the trimeric green bell pepper HPL (*7*). The authors reported two isoforms of HPL possessing molecular weights of 55 kDa when determined by SDS−polyacrylamide gel electrophoresis, and of 170 kDa using size exclusion chromatography, thereby suggesting that both HPL I and II exist as trimers of 55 kDa subunits in their native state. Fractions F1, B, and C (results not shown) also indicated a molecular weight of 170 kDa by size exclusion chromatography, thereby confirming those obtained by Shibata et al. (*7*) who used another purification process including differential centrifugation, ion exchange chromatography, hydroxylapatite chromatography, and gel filtration. HPL from pea seeds (*12*), cucumber (*11*), and tea leaf (*6*) also revealed one predominant band at 55 kDa on SDS−PAGE.

Table 2. Aldehyde Formation by HPO Lyase Preparations

mg aldehydes/ g protein	crude homogenate (60 min bioconversion)	chloroplast fraction (50 min bioconversion)	purified HPO lyase (20 min bioconversion)
hexanal	21.15	159.62	
trans-2-hexenal	67.17	238.25	7500
total	88.32	391.87	7500

Chloroplasts Isolation. From 150 g of green bell pepper, 2 g of chloroplasts were obtained. Optical microscopic observation of the chloroplastic fraction showed that this fraction was composed of almost pure chloroplasts and a few other cell components. This fraction was further used for the bioconversion assays.

Plant organelles such as chloroplasts are the site of biosynthesis for regulatory molecules potentially involved in plant defense reactions. LOX, HPO lyase, and dehydratase activities have been previously measured in chloroplast preparations (*2*, *19*).

It has now been well established that HPO lyase is a membrane-bound enzyme. Indeed, chloroplasts from cucumber peel (*35*), tea leaves (*36*), endive (*22*), and spinach leaves (*19*) have been shown to have high HPO lyase activities. More recently, HPO lyase activity from olive fruits has been associated with the high-density fraction obtained after centrifugation, and a high correlation was found with the chlorophyll content (*10*).

Moreover, the hydroperoxide metabolism is associated with the external envelope membranes of chloroplasts and not with the thylakoides (*20*).

C6-Aldehydes Formation. In this study, a mixture of the 13-HPODs and 13-HPOTs obtained from the oxygenation of the linoleic and linolenic acids present in olein LE 80 by soybean flour lipoxygenase was used as substrate. For the enzyme assays using the three different HPO lyase preparations, *trans*-2-hexenal was the predominant product. These results may be due to a preferential substrate specificity of green bell pepper HPO lyase for 13-HPOT. Indeed, the 13-HPOT has been shown to be the best substrate for both the HPL I and II isoenzymes, and the activities were about 12 times higher with 13-HPOT than those observed with 13-HPOD (*7*). Olias et al. (*5*) reported that the HPO lyase of soybean seedlings had no activity with the 9-HPODs and 9-HPOTs as substrates, and that 13-HPOD was a better substrate than 13-HPOT.

With the crude homogenate (**Table 2**), the maximum formation of 21.15 mg of hexanal/g protein and of 67.2 mg *trans*-2-hexenal/g protein was reached within 60 min. This value was quite low compared to those obtained with the chloroplast fraction (159.62 mg hexanal/g protein and 238.25 mg *trans*-2-hexenal/g protein respectively) and could be due to either inhibitions caused by substances present in the crude homogenate or the presence of other HPO-degrading activities present in the crude homogenate.

For the chloroplast fraction, the maximum formation of 159.62 mg of hexanal and 238.25 mg of *trans*-2-hexenal per g of protein was reached within 50 min (**Table 2**). These values were 7.5 and 3.5 times higher for hexanal and *trans*-2-hexenal, respectively, than those found with the crude homogenate. The purification process used to obtain the chloroplast fraction could, therefore, have removed the soluble inhibitors present in the crude extract, thereby resulting in a higher HPL activity; however, the other HPO-degrading activities localized in the chloroplast membranes (*20*) could have remained present.

With the purified enzyme (fraction FI), the maximum formation of *trans*-2-hexenal (7,500 mg/g protein) was reached within 20 min of bioconversion, which was much more than that obtained with the crude and chloroplast preparations of HPO lyase. However, no hexanal was detected in these conditions. These results suggest that only one isozyme of HPO lyase specific for 13-HPOT was purified using this purification process. A major advantage of this purification process should be the elimination of all the other HPO-degrading activities already described by Blee and Joyard (*20*) to be restricted to the envelope membranes of plastids. Moreover, all the inhibitors (lipophilic antioxidant, inhibitors with phenolic hydroxy groups, etc.) (*7*) potentially present in the crude homogenate were removed by this purification process.

However, for all three enzymatic preparations, "the suicide-like" inhibition of HPO lyase already described (*37*), could have occurred to different extents. This inhibition does not seem to be caused by the direct products of the reaction (hexanal, *trans*-2-hexenal, and 12-oxo-9-decenoic acid) (*34*). These authors reported that tea leaf HPO lyase was inhibited progressively by its substrates, especially 13-HPOT, and that the inactivation rate was correlated to the hydroperoxide concentration. The presence of a sulfhydryl group in the active center of various hydroperoxide lyases (*38*, *6*, *7*) has been demonstrated by inhibition experiments using Hg^{2+} ions or *p*-chloromercuryphenyl-sulfonic acid. These hydroperoxides could therefore be introduced into the HPO lyase reactional center and subsequently converted into a reactive radicalar species, thereby destroying the enzyme's thiol group, essential for its catalytic activity. In support of this radical reaction, inhibition of enzymatic activity by radical scavengers was observed, and the protective effect of radical scavengers against inactivation of HPO lyase by its substrates was demonstrated (*18*).

In conclusion, three preparations of HPO lyase from green bell pepper were used in this study in order to compare their efficiency in producing green notes aldehydes.

The increase of the aldehyde production observed with the chloroplast fraction is interesting, because it is not a very time-consuming process. From a technical point of view, the recovery of the chloroplasts implies two supplementary centrifugations compared to the preparation of the crude homogenate. Moreover, the use of the chloroplast fraction of green bell pepper seems to be a good compromise between use of the crude homogenate and use of the purified fraction to develop efficient and economical industrial-scale processes to produce these aldehydes. This process could be improved further by immobilization of this fraction.

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