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Formation of Volatile Compounds in Model Experiments with Crude Leek (*Allium ampeloprasum* Var. *Lancelot*) Enzyme Extract and Linoleic Acid or Linolenic Acid

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Three continuous assays are described for lipoxygenase (LOX), hydroperoxide lyase (HPL) and alcohol dehydrogenase (ADH) in leek tissue. The catalytic activity of LOX showed significant difference (significance level 5%) between linolenic acid (9.43×10^{-4} katals per kg protein) and linoleic acid (2.53×10^{-4} katals per kg protein), and the pH-optimum of LOX was 4.5−5.5 against linoleic acid. The catalytic activity of HPL was statistically the same for 9-(S)-hydroperoxy-(10E,12Z)-octadecadienoic acid (1.01×10^{-2} katals per kg protein) and 13-(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid (7.69×10^{-3} katals per kg protein). ADH showed a catalytic activity of 5.01×10^{-4} katals/kg of protein toward hexanal. Model experiments with crude enzyme extract from leek mixed with linoleic acid or linolenic acid demonstrated differences in the amount of produced aroma compounds. Linoleic acid resulted in significantly most hexanal, heptanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-decadienal, pentanol, and hexanol, whereas linolenic acid resulted in significantly most (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*,*Z*)-2,4-heptadienal, (*E*,*E*)-2,4-heptadienal, and butanol. Leek LOX produced only the 13-hydroperoxide of linoleic acid and linolenic acid.

KEYWORDS: Leek; flavor; lipoxygenase; hydroperoxide lyase; alcohol dehydrogenase; enzyme assays

INTRODUCTION

The aroma of freshly cut leek and other *Allium* species is dominated by numerous sulfur containing volatile compounds originating from decomposition of the odorless nonvolatile precursors S-alk(en)yl-cysteine sulfoxides by action of alliinase (EC 4.4.1.4) (*1*, *2*). However, volatiles produced by the lipoxygenase pathway also contribute to the aroma profile of freshly cut leeks (*3*) and especially of frozen stored leek slices (*4*). The lipoxygenase pathway in plants is very complex and over hundred products from lipoxygenase-generated hydroperoxides of linoleic acid have been reported (*5*). In the plant the catalyzed reactions are involved in defense, senescence, seed germination, stress response, and communication (*6*−*8*), and some of these reactions also lead to the formation of short-chain aldehydes and alcohols, which will contribute to the aroma of freshly cut leeks. Production of these aroma compounds could also be possible by autoxidation (*9*).

Lipoxygenases (EC 1.13.11.12) (LOX) are non-heme, iron-containing dioxygenases that catalyze the formation of hydroperoxy derivatives of polyunsaturated fatty acids with a *cis,cis*-pentadiene moiety (*7*, *10*) In plants, the most common substrates are linoleic acid and linolenic acid (*7*). Dioxygen is introduced either at the 9- or 13-carbon of linoleic or linolenic acid leading to the formation of the corresponding conjugated hydroperoxy-diene or -triene fatty acid. The ratio of 9- to 13-hydroperoxides is depending on the origin of LOX (*5*, *7*).

Volatile aldehydes and nonvolatile oxoacids are produced by the action of hydroperoxide lyase (HPL) on the formed hydroperoxides (*7*, *11*). Likewise with LOX, there are different types of HPL classified according to their substrate specificity (*5*, *12*, *13*). One isoenzyme is specific toward the 9-hydroperoxide, one toward the 13-hydroperoxide and one is nonspecific and therefore cleaving both hydroperoxides (*13*). When metabolizing linolenic acid hydroperoxide, HPL retains the *Z*-configuration of the double bond from the hydroperoxide to the aldehyde, but isomerization by isomerases to the *E*-isomer occurs after the cleavage reaction (*12*). All of these aldehydes, both saturated and nonsaturated, can be further metabolized by alcohol dehydrogenase (EC 1.1.1.1) (ADH) to the corresponding alcohols by consumption of NADH (*11*).

Few investigations have been carried out on LOX, HPL, or ADH originating from leek or other *Allium* and therefore little is known about the potential substrate specificity for LOX and HPL in leek. Clayes et al. (*14*) have found lipoxygenase metabolites in onions, but they did not investigate the activity of lipoxygenase. Investigations have been made on the LOX

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activity in leek (*4*, *15*, *16*), onions and garlic (*17*), and chives (*18*), but only refs *4* and *16* were related to aroma formation.

The aim of this paper was to develop and/or optimize three continuous assays for analyzing activities of LOX, HPL, and ADH in leek. One assay was based on measuring oxygen consumption and two assays were based on the oxidation of NADH measured spectrophotometrically. Also the nature of LOX and HPL originating from leek was investigated with reference to substrate specificity and determination of pH-optimum of LOX. Finally, this paper looks into the formation of aroma compounds when crude enzyme extract from leek are mixed with linoleic acid or linolenic acid.

MATERIALS AND METHODS

Plant Material. Leeks (*Allium ampeloprasum*, Var. *Bulga*) were harvested fully matured at Funen, Denmark in October 2001 and used for the optimization of the enzyme assays immediately after. Leeks (*Allium ampeloprasum*, Var. *Lancelot*) were bought in a local store and used for the aroma model experiment immediately after.

Chemicals. Reference compounds (purity ≥ 97%): Propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol were bought commercially from Sigma-Aldrich, Copenhagen, Denmark. (*E*,*Z*)-2,4-Heptadienal was occurring as an impurity in (*E*,*E*)-2,4-heptadienal. All other chemicals, except 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid, were of analytical grade and were bought commercially from Sigma-Aldrich.

Synthesis of 9-(S)-Hydroperoxy-(10*E*,12*Z*)-octadecadienoic Acid (9-HPODE). Sodium linoleate (1.25 mL, 10mM), 4.75 mL of air saturated 50 mM potassium phosphate buffer (pH 7.0) and 1.50 mL of potato tuber lipoxygenase solution were mixed then agitated and purged with oxygen for 30 min. The solution was checked for absorbance at 234 nm to determine the content of 9-HPODE. According to Galliard and Phillips, (*19*) potato tuber LOX almost exclusively converts linoleic acid into 9-HPODE.

Preparation of Potato Tuber Lipoxygenase Solution for Synthesis. Grated potato tubers (50 g) and 50 mL of tap water were homogenized for 30 s in a Waring commercial blender and filtrated through a paper filter. A 2-mL aliquot of the liquid was applied to a DEAD-Cellulose C545 column (anion exchanger) followed by 6.0 mL of 50 mM potassium phosphate buffer (pH 7.0). Two fractions of 4.0 mL were collected; both were checked for activity, and the second one was used as the lipoxygenase solution.

Preparation of Enzyme Extract. The white part of the leek stem was cut into 4-mm slices. Enzyme extract was made by mixing 200 g of leek slices with 200 mL of potassium phosphate buffer (50 mM, pH 7.0 added 0.1% Triton X-100) for 45 s in a Waring commercial blender. The slurry was kept on ice with agitation for 30 min (100 rpm) and afterward filtered through a paper filter until 75.0 mL was collected. Seven of these extractions were mixed, frozen, and used as the enzyme source through all experiments.

Experimental Design. Crude enzyme extract from leeks was analyzed for catalytic activity of LOX, HPL, and ADH in triplicate. Substrate specificity of LOX toward linoleic acid and linolenic acid was investigated, and the pH-optimum was determined. Also, substrate specificity of HPL toward 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid (9-HPODE) and 13-(S)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13-HPODE) was investigated.

The enzyme extract was mixed with linoleic acid or linolenic acid or phosphate buffer (blank samples) and left for 2, 10, 30, or 60 min to react prior to aroma analysis. Blank samples were also run on the fatty acids alone with a reaction time of 60 min.

Lipoxygenase Assay. LOX was assayed in a continuous assay by measuring consumption of initial dioxygen by an YSI 5100 dissolved oxygen meter (YSI Inc., Yellow Springs, OH) at 30 °C, using linoleic acid or linolenic acid as the other substrate. Calibration was done at 30 °C by air-saturated tempered phosphate buffer (0.2 M, pH 6.0) (21% dissolved dioxygen) followed by addition of sodium dithionite (0% dissolved dioxygen). To determine lipoxygenase activity, 27.2 mL of air-saturated phosphate buffer (0.2 M, pH = 6.0) and 3.9 mL of enzyme extract was mixed in a 33-mL conical flask and the measuring was started. After 30 s, the reaction was initiated by adding 1.9 mL of sodium linoleate solution (10 mM). Dioxygen was monitored over 20 min, measuring dioxygen content every second.

Blank samples were run on buffer added substrate or enzyme extract.

Calculation of LOX Activity. LOX activity was calculated as katals per kg of protein, and katals was defined as moles of O_2 consumed per second. This was done under the assumption that air-saturated phosphate buffer (0.2 M, pH 6.0) at 30 °C with a salt strength of 12.40 g/L contains 7.11 mg O_2/L(*20*). To determine the activity, the dioxygen level (millimoles of O_2) was plotted against time, and the steepest slope (millimoles of O_2 per second) on the curve was found using continuous linear regression over 20 s at a time.

Determination of pH-Optimum of LOX. pH-Optimum was examined by triplicate determinations of the catalytic activity of LOX against linoleic acid in 0.1 M acetate buffer with the following pH values: 3.5, 4.0, 4.5, 5.0, and 5.5 and in 0.2 M phosphate buffers with the following pH values: 6.0, 6.5, 7.0, and 7.5. Blank samples were run at each pH value.

Hydroperoxide Lyase Assay. HPL was assayed in a continuous coupled assay modified after (*21*). ADH converts the products of HPL's reaction on hydroperoxides, aldehydes, under the oxidation of NADH, which can be monitored spectrophotometrically at 340 nm.

Phosphate buffer (1.8 mL, 0.2 M, pH = 6.0), 300 μL of 2.2 mM NADH, 200 μL of ADH (150 units in 100 μL) and 300 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific, Braine-l'Alleud, Belgium) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 400 μl of 9-HPODE or 13-HPODE was added. The reaction was monitored over 600 s measuring every second.

Blank samples were run on buffer, NADH, and ADH with substrate or enzyme extract.

Calculation of HPL Activity. HPL activity was calculated as katals per kg of protein, and katals was defined as moles of hydroperoxide consumed per second. To determine the activity, the absorbance was plotted against time, and the steepest slope (absorbance/s) on the curve was found using continuous linear regression over 5 s at a time. Absorbance was converted to moles of hydroperoxide by the assumption that each mole of hydroperoxide metabolized produced one mole of aldehyde. Aldehydes are converted by alcohol dehydrogenase by the consumption of NADH 1:1. ϵ of NADH at 340 nm is 6220 L/mol · cm (*21*).

Alcohol Dehydrogenase Assay. ADH was assayed in a continuous assay by following the oxidation of NADH spectrophotometrically at 340 nm when hexanal was added.

Borate buffer (1.4 mL, 0.05 M, pH = 9.0), 300 μl of 2.2 mM NADH and 1000 μL of enzyme extract (filtrated through 1.2-μm and 0.45-μm filters from Orange Scientific) were transferred to a thermostatic (30 °C) 1-cm cuvette with a magnetic stirrer, and the measuring was started. After 30 s, 300 μL of hexanal (100 ppm) was added. The reaction was monitored over 600 s measuring every second.

Blank samples were run on buffer and NADH with substrate or enzyme extract.

Calculation of ADH Activity. Alcohol dehydrogenase activity was calculated as katals per kg of protein, and katals was defined as moles of hexanal consumed per second. Moles of hexanal were calculated on the basis of NADH under the assumption that hexanal and NADH were metabolized 1:1 and by the conditions described under calculation of HPL activity.

Determination of Protein Content. Protein content in the enzyme extraction was determined by Coomassie brilliant blue, according to the method described in US/EG Bulletin 1069 from Bio-Rad Life Science Group, Hercules, CA, using lyophilized bovine serum albumin as the standard protein.

Model Experiments. Air-saturated phosphate buffer (60.0 mL, 0.2 M, pH 6.0), 8.0 mL of enzyme extract and 4.0 mL of sodium linoleate solution (10 mM) or sodium linolenate solution (10 mM) were mixed in a closed 500-mL glass flask at 30 °C with agitation (200 rpm) for 2, 10, 30, or 60 min. Immediately after the reaction period, 3.0 g of

Table 1. Catalytic Activities of Lipoxygenase, Hydroperoxide Lyase, and Alcohol Dehydrogenase in Crude Enzyme Extract of Leek

substrate	lipoxygenase[a]	hydroperoxide lyase[a]	alcohol dehydrogenase[a]
linoleic acid	0.000943 ± 0.00026		
linolenic acid	0.000253 ± 0.000052		
9-HPODE[b]		0.0101 ± 0.0036	
13-HPODE[c]		0.00769 ± 0.0022	
hexanal			0.000501 ± 0.000093

[a] All values are in katal/kg protein. [b] 9-(S)-Hydroperoxy-(10E,12Z)-octadecadienoic acid. [c] 13-(S)-Hydroperoxy-(9Z,11E)-octadecadienoic acid.

calcium chloride was added with agitation to inactivate enzymes. Aroma analysis was performed directly after the saturation.

Blank samples were made with enzyme extract with 4 mL of phosphate buffer instead of the fatty acids or with each of the fatty acids with 8 mL of phosphate buffer instead of the enzyme extract. Blank samples were treated as described above, but only a reaction time of 60 min was applied.

Dynamic Headspace Analysis. Aroma compounds were isolated by dynamic headspace with nitrogen (purity = 99.8%) as purge gas. Internal standard (1.0 mL, 50 ppm 4-methyl-1-pentanol in tap water) was added to the solution in the 500-mL glass flask, which was left for 5 min at 30 °C with agitation (200 rpm) to equilibrate the temperature before purging. Aroma compounds were trapped on 250 mg Tenax GR (mesh size = 60/80, Buchem bv, Apeldoorn, The Nederlands) for 45 min with a nitrogen flow of 100 mL/min and agitation (200 rpm).

Desorption of aroma compounds was done thermally by an ATD 400 automatic thermal desorption system (Perkin-Elmer, England). Desorption temperature of the trap to the cold trap (contains 30 mg Tenax GR, temp 5 °C) was 250 °C for 15 min with a helium flow of 60 mL/min. Desorption temperature of the cold trap was 300 °C for 4 min, with a helium flow of 11 mL/min and an outlet split ratio of 1:10. Separation was performed by a GC-MS (HP G1800 A GCD system) with the following conditions: column, DB Wax from J&W Scientific (30-m × 0.25-mm i.d. × 0.25-μm film thickness); carrier gas, helium; start flow, 1 mL/min; split ratio, none; column pressure, 48 kPa (constant); oven program, 45 °C for 10 min, 6 °C/min to 240 °C, constant at 240 °C for 30 min. The mass selective detector was operated in the electron ionization mode (ionization energy, 70 eV), and the *m*/*z* (mass/charge) ratio ranged between 10 and 450. Identification was done by probability-based matching with mass spectra in the G1035A Wiley library (Hewlett-Packard). Identity was confirmed for all detected compounds by checking with mass spectra and retention indices obtained in the laboratory from reference compounds.

Quantification. Aliquots of 10 μL of the reference compounds propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal, (*E*)-2-butenal, (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol were each dissolved in 20 mL of 96% ethanol and diluted with tap water into three appropriate concentrations depending on the concentration of the compounds in the model experiments. Dynamic headspace analysis was performed on 72.0 mL of the solutions with 1.0 mL of internal standard (50 ppm 4-methyl-1-pentanol in tap water) with the same flow, time, and temperature conditions as applied to the model experiments. Each reference compound was analyzed in triplicate in all three concentrations. The obtained peak area divided by the peak area of the internal standard was used to calculate the concentration of the same compound in the model experiments from the peak area of the compound divided by the peak area of the internal standard.

The retention time indices (RTI) of the volatile compounds were calculated with a mixture of hydrocarbons (C_9−C_{26}) as references, according to the method of (*22*).

Statistical Analysis. Analysis of variance was carried out on each of the aroma compounds using the one-way and the factorial ANOVA procedure in the Analyst part of SAS, version 8.2, SAS institute Inc., Cary, NC. The effect of the reaction period and the type of substrate was tested. Sample means were compared by Duncan's multiple range test. A significance level of 5% was applied. The effect of pH on LOX activity and type of substrate on LOX and HPL was tested by the one-way ANOVA procedure and by Duncan's multiple range test (significance level 5%).

RESULTS AND DISCUSSION

To find an extraction method suitable for all three enzymes, extractions were made with phosphate buffer (0.2 M, pH = 6.0) added 0.1% Triton X-100, with acetate buffer (0.1 M, pH = 6.0), with tap water, or with 0.1 M $CaCl_2$ solution because of the possible calcium requirements of LOX isoenzymes. HPL is difficult to isolate because of its binding to membranes (*5*, *23*), probably to the thylakoid membrane of chloroplasts (*24*), and a detergent might be needed for the solubilization (*25*). Also, the effect of the extraction time was tested between 0 and 60 min. These experiments demonstrated that the most suitable method all together was to use phosphate buffer (0.2 M, pH = 6.0) with 0.1% Triton X-100 as the extraction fluid and to apply an extraction time of 30 min.

Table 1 shows the catalytic activities of LOX, HPL, and ADH measured in the crude enzyme extract. Measurements of LOX activity on the two fatty acids proved a significant difference, linoleic acid resulted in almost 4 times higher activity than linolenic acid. HPL activity was also checked on two substrates, the 9-hydroperoxide and the 13-hydroperoxide of linoleic acid. In most plants, HPL is specific for one of the hydroperoxides (*26*), but in this case, there was no statistical difference between the two substrates. This indicated that HPL from leek was capable of transforming both hydroperoxides or that two or three HPL isoenzymes were present. Husson and Belin (*26*) found two isoforms of HPL in green bell pepper but did not investigate the activity toward 9-hydroperoxides. The activity of ADH was tested with hexanal as substrate and was found to be 5.01 × 10^{-4} katals/kg of protein.

The pH-optimum of LOX in leek was 4.5−5.5 with linoleic acid as substrate (**Figure 1**). pH-Optimum with linolenic acid as substrate was not examined. We have not found any other investigations relating to pH-optimum of LOX in leeks, but Egert and Tevini (*18*) found that LOX activity in chives has a pH-optimum at pH 6, and investigations on potato LOX demonstrated pH-optimum at pH 5.5−6.0 (*27*,*28*). We found that the length of the lag phase was not influenced by the pH change (data not shown); the values were 50−150 s (not statistically different) but with no correlation to the pH change.

Table 2 displays all of the aldehydes and alcohols found in the model experiments of the two fatty acids, the enzyme extract without fatty acid added (blank), and the blank measurements of linoleic acid and linolenic acid after a reaction time of 60 min (autoxidation controls). Many sulfur compounds were detected as well, but as they are not relevant for the concern of this paper, the results were not shown or discussed in details. One thiol (1-propanethiol) was detected in minor concentrations in the enzyme extract, and although thiols can react with the carbonyl group in the aldehydes, this would occur in equal amounts in the three samples with enzyme extract and consequently not influence the result.

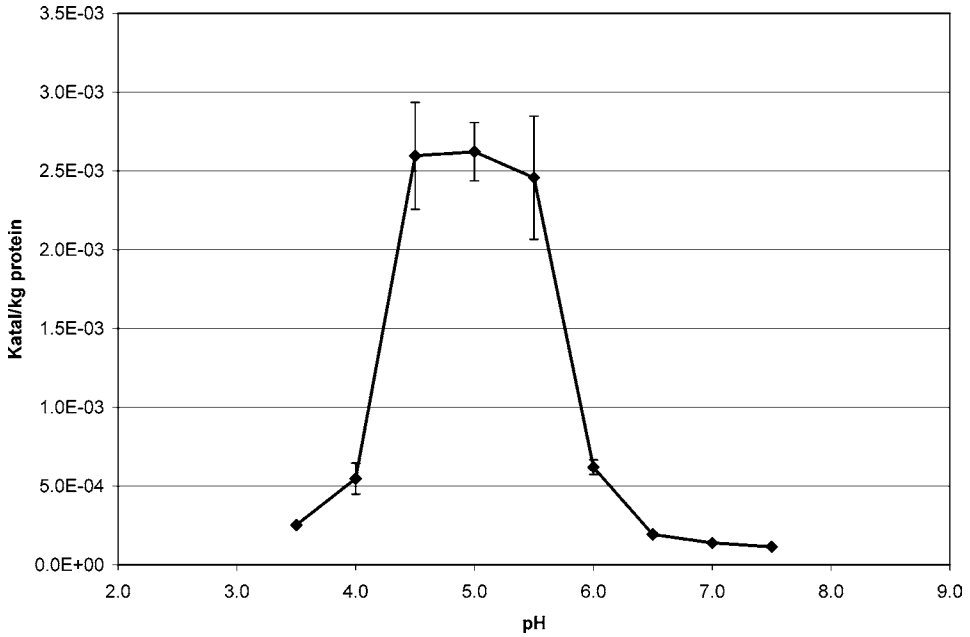

Figure 1. pH response curve for LOX activity against linoleic acid substrate in crude leek extract. Vertical bars indicate standard deviation.

Table 2. Aldehydes and Alcohols Found in the Model Experiments of the Leek Enzyme Extract with Either Linoleic Acid, Linolenic Acid, or No Fatty Acid (Enzyme Blank), or Fatty Acid without Leek Enzyme Extract (Acid Blanks) after a Reaction Time of 60 min

compound	RTI[a]	linoleic acid + enzymes[b]	linolenic acid + enzymes[b]	enzyme blank[b]	linoleic acid blank[b]	linolenic acid blank[b]
aldehydes						
propanal	762	0.838 ± 0.12	0.916 ± 0.057	0.761 ± 0.22	0.00 ± 0	0.147 ± 0.0057
butanal	867	0.00463 ± 0.00016	0.00459 ± 0.00037	0.00165 ± 0.00028	0.000293 ± 0.000027	0.00251 ± 0.00029
pentanal	984	0.162 ± 0.015	0.163 ± 0.0084	0.185 ± 0.021	0.00 ± 0	0.0177 ± 0.00090
hexanal	1111	0.895 ± 0.025	0.0721 ± 0.0045	0.130 ± 0.0072	0.00482 ± 0.00058	0.00 ± 0
heptanal	1197	0.00554 ± 0.00012	0.00232 ± 0.00025	0.00378 ± 0.00076	0.000688 ± 0.00016	0.00107 ± 0.00026
octanal	1299	0.000332 ± 0.0000044	0.000525 ± 0.000052	0.000808 ± 0.000031	0.000242 ± 0.000123	0.000409 ± 0.000040
nonanal	1398	0.000900 ± 0.00011	0.00164 ± 0.00026	0.00288 ± 0.000094	0.000258 ± 0.000027	0.000300 ± 0.000080
(*E*)–2-butenal	1046	0.00238 ± 0.00018	0.00995 ± 0.00048	0.0150 ± 0.0007	0.00 ± 0	0.00 ± 0
(*E*)–2-pentenal	1147	0.0161 ± 0.0011	0.0909 ± 0.00015	0.0236 ± 0.00070	0.00 ± 0	0.00 ± 0
(*E*)–2-hexenal	1230	0.00446 ± 0.000087	0.0073 ± 0.0010	0.00230 ± 0.000072	0.00 ± 0	0.00 ± 0
(*E*)–2-heptenal	1334	0.0384 ± 0.0012	0.0024 ± 0.00014	0.00438 ± 0.000029	0.00 ± 0	0.00 ± 0
(*E*)–2-octenal	1434	0.0345 ± 0.0014	0.00105 ± 0.00012	0.00191 ± 0.00094	0.00 ± 0	0.00 ± 0
(*E*,*Z*)–2,4-heptadienal	1464	0.00339 ± 0.00063	0.0151 ± 0.0022	0.00 ± 0	0.00 ± 0	0.00 ± 0
(*E*,*E*)–2,4-heptadienal	1490	0.00363 ± 0.00023	0.0118 ± 0.0011	0.000914 ± 0.000081	0.00 ± 0	0.00 ± 0
(*E*,*E*)–2,4-decadienal	1789	0.0575 ± 0.0029	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.00 ± 0
alcohols						
1-propanol	1049	0.0156 ± 0.0019	0.0176 ± 0.0023	0.0147 ± 0.0014	0.00 ± 0	0.00 ± 0
1-butanol	1166	0.000959 ± 0.000025	0.00401 ± 0.000071	0.00142 ± 0.00012	0.00 ± 0	0.00 ± 0
1-pentanol	1274	0.0339 ± 0.00074	0.0108 ± 0.00031	0.0125 ± 0.00160	0.00 ± 0	0.00 ± 0
1-hexanol	1371	0.00640 ± 0.00053	0.00256 ± 0.000077	0.00331 ± 0.00014	0.00 ± 0	0.00 ± 0

[a] Retention time index. [b] All values except RTI are in mg/L ± standard deviation.

When the amount of aldehydes and alcohols obtained from enzymatic treatment of the two fatty acids after a reaction time of 60 min. was compared with the results from the enzyme extract blank (**Table 2**), there were evident differences. Linoleic acid led to formation of significantly mostly hexanal, heptanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-decadienal, pentanol, and hexanol, whereas linolenic acid resulted in significantly mostly (*E*)-2-pentenal, (*E*)-2-hexenal, (*E*,*Z*)-2,4-heptadienal, (*E*,*E*)-2,4-heptadienal, and butanol. Butanal was produced in equal amounts in the two fatty acid samples but in higher concentration than the enzyme blank sample, whereas propanal, pentanal, and 1-propanol were produced in the same quantities in the two fatty acid samples and the enzyme blank sample. Octanal, nonanal, and (*E*)-2-butenal were significantly highest in the enzyme blank sample.

As seen in **Table 2,** most of the volatile compounds were not present in the linoleic acid blank and the linolenic acid blank samples; exceptions were butanal, hexanal, heptanal, octanal, and nonanal in linoleic acid and propanal, butanal, pentanal, heptanal, octanal, and nonanal in linolenic acid. However, these were produced in a significantly lower level than they were in the samples with the fatty acids added enzymes.

According to Hornostaj and Robinson (*13*) the cleavage of the 9-hydroperoxides of linoleic acid and linolenic acid by HPL are converted to (*Z*)-3-nonenal and (*Z*,*Z*)-3,6-nonadienal, respectively, whereas the cleavage of the 13-hydroperoxide yields hexanal and (*Z*)-3-hexenal, respectively. These aldehydes are further converted by the action of ADH into the corresponding alcohols or by isomerization of the (*Z*)-3-enals to (*E*)-2-enals by isomerases (*10*). Hexanal (**Figure 2**) was developing all

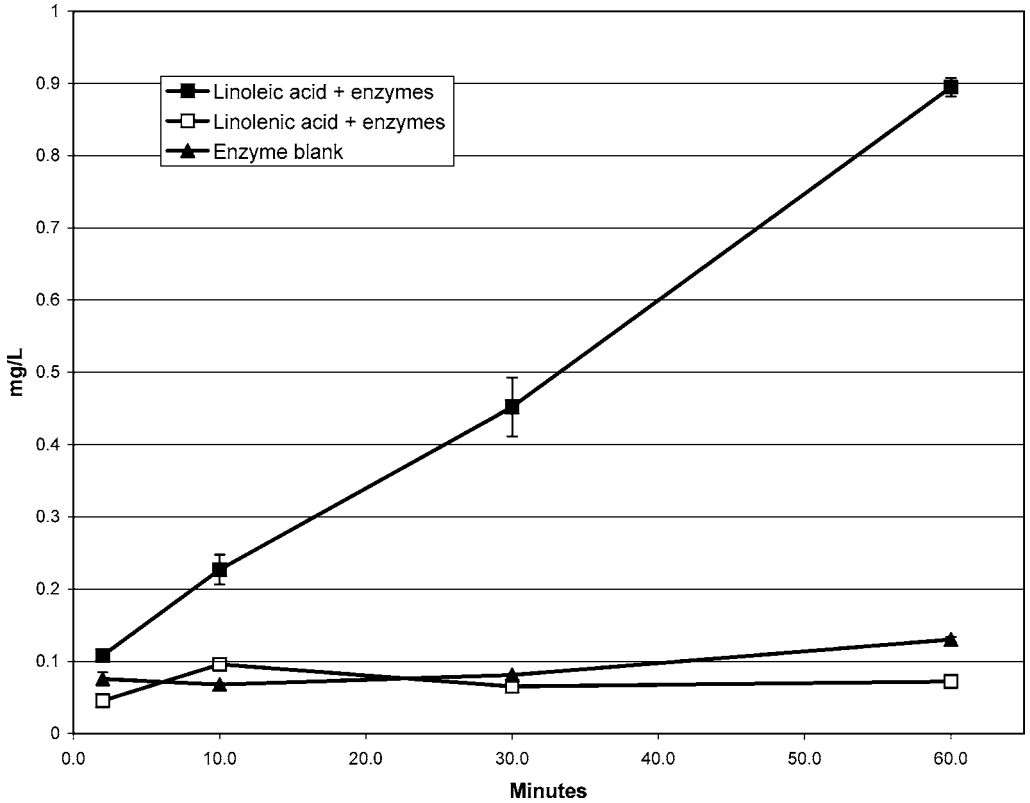

Figure 2. Development of hexanal during 60 min reaction in the model experiments of crude leek enzyme extract with linoleic acid or linolenic acid as substrate or without added fatty acid (enzyme blank). Vertical bars indicate standard deviation.

through the reaction time of 60 min when linoleic acid substrate was added, whereas the blank samples and the linolenic acid substrate showed no development over time, which confirmed the fact that hexanal is produced from the 13-hydroperoxide of linoleic acid (*29*). (*E*)-2-hexenal, which originates from the 13-hydroperoxide of linolenic acid, also showed statistical differences between the two fatty acids and the blank samples when the whole time range was considered (data not shown). In this case, linolenic acid resulted in the highest production of (*E*)-2-hexenal, but linoleic acid also showed an increase in (*E*)-2-hexenal compared to the enzyme blank. (*E*)-2-pentenal also showed the pattern described for (*E*)-2-hexenal, except that adding linoleic acid gave only a minor production of (*E*)-2-pentenal. This is consistent with Gardner et al. (*30*), who describe the formation of (*E*)-2-pentenal from the 13-hydroperoxide of linolenic acid by the action of LOX and ADH in soybean. (*E*,*E*)-2,4-Heptadienal and (*E*,*Z*)-2,4-heptadienal showed the same development.

The present aroma results indicated that only the 13-hydroperoxide of linoleic acid was produced, because only hexanal and not (*E*)-2-nonenal was found when linoleic acid was added. Likewise, only (*E*)-2-hexenal and not 3,6-nonadienal was found when linolenic acid was added. According to Gardner (*5*), LOX with pH-optimum near neutrality is normally specific toward 9-(S)-oxidations, while pH-optimum close to 9 usually results in specificity toward 13-(S)-oxidations. Our results could not confirm this, as pH-optimum was determined to be 4.5−5.5. The results of the aroma analysis also indicated that LOX was metabolizing linoleic acid more readily than linolenic acid, because production of hexanal when linoleic acid was added was more than 100 times greater than the production of (*E*)-2-hexenal when linoleic acid was added. The same trend was found for the catalytic activity of LOX expressed as moles of O_2 consumed per second toward the two substrates.

(*E*)-2-Heptenal and (*E*,*E*)-2,4-decadienal (**Figure 3**) showed the same development as hexanal, except that (*E*,*E*)-2,4-decadienal was not detected in the linolenic acid sample and the three blank samples. This indicated that the production was caused by enzymatic activity and not by autoxidation.

The addition of linoleic acid to the enzyme extract led to formation of approximate 30 times more (*E*)-2-octenal than when linolenic acid was added or with no fatty acid added. According to Rosahl (*7*), dioxygen is introduced either at the 9- or 13-carbon by LOX, which means that (*E*)-2-octenal is not a direct product of the lipoxygenase pathway. However, Haslbeck and Grosch (*31*) found that soybean lipoxygenase is also capable of producing the 8-, 10-, 12-, and 14-HPODE as minor components. (*E*)-2-Octenal was formed when the hydroperoxide was placed on the C-10 position of linoleic acid or by autoxidation of (*E*,*E*)-2,4-decadienal (*9*), and experiments with pea LOX showed that (*E*)-2-octenal was formed when linoleic acid was oxidized by LOX (*32*).

Propanal, pentanal, and 1-propanol were produced in statistically equal amounts in the three solutions with enzyme when looking at the whole lapse of time. These apparently did not originate from an enzymatic reaction on one of the two fatty acids and probably not from autoxidation either, because these compounds were not detected at all in the linoleic acid blank and in only minor concentrations (only propanal and pentanal) in the linolenic acid blank. Their origin was most likely from other enzymatic reactions and substrates present in the crude leek extract. Propanal is a breakdown product from the lachrymatory factor thiopropanal-S-oxide originating from alliinase activity (*33*) and 1-propanol can be a result of ADH's action on propanal. Formation of these three aroma compounds had probably already occurred during the preparation of the enzyme extract because of the available substrate in the extract

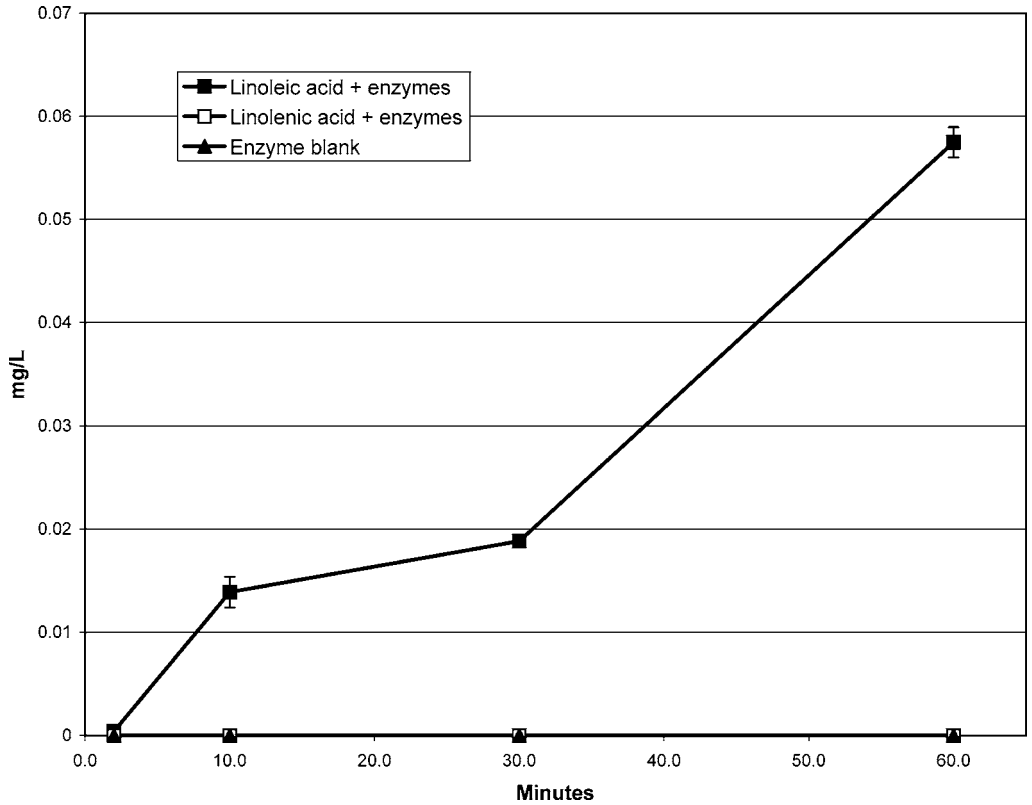

Figure 3. Development of (*E*,*E*)-2,4-decadienal during 60 min reaction in the model experiments of crude leek enzyme extract with linoleic acid or linolenic acid as substrate or without added fatty acid (enzyme blank). Vertical bars indicate standard deviation.

when the cells were ruined, as no further development over the 60 min was observed (data not shown).

Three compounds (octanal, nonanal, and (*E*)-2-butenal) were produced in significantly highest amounts in the enzyme blank. Two of them (octanal and nonanal) were also produced in the two fatty acid blank samples (in a significantly lower level though), which could indicate autoxidation to a certain degree, but there was no increase during the 60 min of reaction. It is not obvious why these three compounds are produced in higher amounts in the enzyme blank than in the fatty acid samples with enzyme extract, unless a decomposition of the compounds was induced when the fatty acids were present. A decrease over time was observed for (*E*)-2-butenal when linoleic acid was added, but linolenic acid resulted in an increase over time.

Four alcohols, 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol, were detected in the three samples with enzyme extract added and not in the fatty acid blank samples, which confirmed that activity of alcohol dehydrogenase was present in the crude leek extract (**Table 1**). Linoleic acid resulted in the highest concentration of 1-pentanol and 1-hexanol, whereas linolenic acid produced the highest amount of 1-butanol.

The model experiments were also performed with microwave heating of the solution to 90 °C (data not shown) instead of calcium chloride inactivation of the enzymes. This method of enzyme inactivation is relevant for the processing of food. The results showed the same trend as that for calcium chloride inactivation, but heat treatment gave higher concentrations of the compounds probably as a result of increased chemical reaction rate and autoxidation. Decanal, (*E*)-2-nonenal, (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-nonadienal, and (*E*,*Z*)-2,4-decadienal were only formed by microwave heating.

LOX originating from leek showed 4 times higher catalytic activity toward linoleic acid than toward linolenic acid. On the basis of the aroma results it, was concluded that leek LOX is specific in the production of hydroperoxides, as no products after the 9-hydroperoxide were detected, and essentially only products after the 13-hydroperoxide were detected. pH-Optimum of leek LOX was found to be 4.5−5.5 against linoleic acid, which was lower than that reported for other plant lipoxygenases. The substrate specificity investigations showed that leek HPL is not specific toward one of the hydroperoxides. A pronounced difference in required reaction time to produce the detected aroma compounds was found, some compounds developed instantly, whereas others still developed after a reaction time of 60 min.

ABBREVIATIONS USED

LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; 9-HPODE, 9-(S)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid; 13-HPODE,13-(S)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid

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