Alfalfa contains substantial 9-hydroperoxide lyase activity and a 3*Z*:2*E*-enal isomerase

Minke A. Noordermeer, Gerrit A. Veldink*, Johannes F.G. Vliegenthart

Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands

Received 23 November 1998; received in revised form 15 December 1998

Abstract Fatty acid hydroperoxides formed by lipoxygenase can be cleaved by hydroperoxide lyase resulting in the formation of short-chain aldehydes and ω-oxo acids. Plant hydroperoxide lyases use 13- or 9-hydroperoxy linoleic and linolenic acid as substrates. Alfalfa (*Medicago sativa* L.) has been reported to contain a hydroperoxide lyase specific for 13-hydroperoxy linoleic and linolenic acid only. However, in addition to 13-hydroperoxide lyase activity we found substantial 9-hydroperoxide lyase activity in alfalfa seedlings as well. The specific activity for 9-hydroperoxy fatty acids was about 50% of the activity for the 13-isomers. Furthermore, alfalfa seedlings contain a 3*Z*:2*E*-enal isomerase that converts the 3*Z*-enal products to their 2*E*-enal isoforms.

Key words: Fatty acid hydroperoxide lyase; 3*Z*:2*E*-enal isomerase; Substrate specificity; *Medicago sativa* L.

1. Introduction

Many higher plants have been reported to be able to produce volatile C6- and C9-compounds such as hexanal, (3*Z*)- and (2*E*)-hexenal, (3*Z*)- and (2*E*)-nonenal, (3*Z*,6*Z*)- and (2*E*,6*Z*)-nonadienal and the corresponding alcohols. These compounds are important constituents of the characteristic flavors of fruits, vegetables and green leaves and are widely used as food additives. Besides, they might also be involved in wound healing and pest resistance [1,2]. They are derived from linoleic and linolenic acids in a catalytic route involving three enzymes. Fatty acids are peroxidized by lipoxygenase and subsequently cleaved by hydroperoxide lyase (HPO lyase). The resulting short-chain aldehydes and ω-oxo acids can be reduced to alcohols by alcohol dehydrogenase. Besides, double bond isomerization can occur. It is still unclear, however, whether this reaction is catalyzed by an isomerase or a non-enzymatic isomerization factor. Although HPO lyase has been purified from different sources, little is yet known about its structure and reaction mechanism. Hatanaka et al. [3] proposed a heterolytic scission mechanism similar to the acid-catalyzed cleavage of linoleate hydroperoxides in an aprotic solvent [4]. However, the recently found homology of HPO lyase with cytochrome P450 enzymes could suggest a homolytic reaction mechanism [5]. Two different types of substrates for plant HPO lyases are known: 13- and 9-hydroperoxy linoleic or linolenic acids. HPO lyases from watermelon seedlings, tea leaves, tomato fruit and leaves, apples, green bell peppers and soybeans are specific for 13-HPO fatty acids [6–11], whereas HPO lyase from pears is specific for 9-HPO fatty acids [12]. Soybean seeds/seedlings and cucumber fruit and seedlings contain both HPO lyase activities [13,14] and Matsui et al. [15] succeeded in separating 13- and 9-HPO lyase from cucumber seedlings. In alfalfa, only 13-HPO lyase was found, and no 9-HPO lyase activity was observed [16–18]. The work presented here, however, provides evidence for the presence of both 13- and 9-HPO lyase activity in alfalfa seedlings, which makes alfalfa interesting for application in a biocatalytic process. Furthermore, a heat-sensitive 3*Z*:2*E*-enal isomerase was found in alfalfa seedlings which could be separated from HPO lyase by anion exchange chromatography.

*Corresponding author. Fax: (31) (30) 2540980.
E-mail: veldink@accu.uu.nl

Abbreviations: HPO, hydroperoxide; 13-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic acid; 13-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid; 9-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid; 9-HPOT, (9*S*,10*E*,12*Z*,15*Z*)-9-hydroperoxy-10,12,15-octadecatrienoic acid; SPME, solid phase micro-extraction; DNPH, 2,4-dinitrophenylhydrazine

2. Materials and methods

2.1. Materials

All chemicals used were commercially obtained and of analytical grade. 13-HPOD and 13-HPOT were prepared from linoleic and α-linolenic acid, respectively (~99%, Fluka Chemie AG, Buchs, Switzerland), with soybean lipoxygenase-1 [19]. Analogously, 9-HPOD and 9-HPOT were prepared with tomato lipoxygenase [20].

2.2. Enzyme activity measurements

HPO lyase activity was determined with the indirect assay described by Vick [21] or by measuring the decrease of the A_{234} due to the cleavage of substrate. One unit of activity corresponds to the amount of enzyme that converts 1 μmol of substrate per min. Protein concentrations were determined with the bicinchoninic acid method after freeze-drying of the samples to remove β-mercaptoethanol [22].

2.3. Enzyme purification

Alfalfa seeds (*Medicago sativa* L.) were purchased from a local nursery. Seeds were soaked in tap water for 6 h and germinated in the dark for 3 days at 20°C. Seedlings were homogenized with 1.7 ml/g buffer A (15 mM phosphate, pH 7.5, 0.2% Triton X-100, 10 mM β-mercaptoethanol) in a blender and filtered through four layers of cheesecloth. The filtrate was centrifuged at 40 000×*g* for 20 min and the lipid top layer was discarded. The supernatant (crude extract) was concentrated by ultrafiltration through a 30 kDa membrane (Diaflo PM30, Amicon Inc., Beverly, MA, USA) and applied to a Sephadex G150 (2.5×23 cm) and a Sepharose Cl4B column (2.5×30 cm) in series (Pharmacia, Uppsala, Sweden).

Elution was performed with buffer B (buffer A with 0.1% Triton X-100 instead of 0.2%) at 0.25 ml/min. The fractions containing HPO lyase activity were pooled and applied to a DEAE Sepharose Cl6B column (1.5×10 cm, Pharmacia, Uppsala, Sweden). Elution was performed at 0.5 ml/min for 8 h with a linear gradient of 0–0.5 M NaCl in buffer B. All purification steps were carried out at 4°C.

2.4. Product identification

For qualitative product analysis 1 U of HPO lyase (determined with 13-HPOD as substrate) was diluted to 8 ml with buffer C (50 mM phosphate, pH 6). 80 μM of substrate was added and the mixture was

PII: S0014-5793(98)01706-2

incubated for 30 min at 20°C in a stirred 20 ml reaction vessel closed with a septum. Headspace compounds were trapped by SPME (100 μm polydimethylsiloxane coated fiber, Supelco Inc., Bellefonte, USA) and desorbed at 200°C for 1 min in the injection port of a GC/FID (HP-Innowax column; 0.25 μm film thickness, 30 m×0.32 mm, Hewlett-Packard). The temperature program used was 35–200°C, 10°C/min with a start and end isotime of 2 min. Non-volatile compounds were extracted from the reaction mixture, previously acidified with HCl to pH 5, with an octadecyl solid phase extraction column (J.T. Baker B.V., Deventer, The Netherlands) and eluted with 100% methanol. The compounds were reduced with an excess of $NaBH_4$ at 0°C, esterified with ethereal diazomethane, silylated with silylating reagent (pyridine/1,1,1,3,3,3,-hexamethyldisilazane/chlorotrimethylsilane 5/1/1 v/v/v) and analyzed with GC/MS (Fisons GC 8000 series and Fisons Instruments MD 800 MassLab spectrometer, CP-Sil5 CB-MS column, 0.25 μm film thickness, 25 m×0.25 mm, Chrompack). The temperature program used was 140–280°C, 6°C/min with a start and end isotime of 2 min. Electron impact mass spectra were recorded with an ionization energy of 70 eV.

Quantitative product analysis was performed with HPLC after derivatization with DNPH. 0.5 U of enzyme was diluted to 5 ml with buffer C and incubated with 80 μM of substrate in a stirred vessel at 20°C. After different time periods the reaction was stopped by addition of 5 ml ethanol containing 0.1% DNPH and 0.5 M HAc. 200 μl of 2.5 mM octanal in isopropanol/water (1/1) were added as an internal standard. After 30 min the reaction products were extracted with 2×4 ml hexane, the hexane was evaporated and the products were dissolved in 0.5 ml methanol. HPLC was performed with a C18 column (Chromsphere 5, 250×4.6 mm, Chrompack) and acetonitrile/THF/water (80/1/19, 1 ml/min) as eluent. The products were quantified by measuring the absorption at 350 nm.

3. Results and discussion

The substrate specificity of alfalfa HPO lyase was determined with the assay described by Vick [21], in order to distinguish between HPO lyase and other hydroperoxide converting enzymes. The results are shown in Table 1. A crude extract of alfalfa seedlings was used to prevent possible changes in the ratios of the activities for the different substrates caused by purification steps. Although HPO lyase activity is highest for the 13-isomers, there is substantial 9-HPO lyase activity as well. This is in contrast to previous findings where no 9-HPO lyase activity was observed in alfalfa [16–18]. In the latter studies however, HPO lyase activity was determined by measuring the amount of aldehydes formed by GLC of essential oils. It might be possible that the C9-aldehydes were not observed because of their reduced vapor pressure compared to the C6-aldehydes. In this paper we show that it is possible to analyze C9-aldehydes with SPME-GC, because they are strongly absorbed to the polydimethylsiloxane coated fiber. Furthermore, alfalfa HPO lyase has a slight preference for the hydroperoxytrienes derived from linolenic acid compared to the hydroperoxydienes derived from linoleic acid. The enzymatic pH optimum was determined and appeared to be equal for 13- and 9-hydroperoxy fatty acids, namely pH 5.5. The pH optimum of alfalfa HPO lyase is in accordance with the optimal pH values described for HPO lyases from other origins, which range from 5.5 in tomatoes and green bell pepper fruit to 8 in cucumber fruits [8,9,15]. However, in contrast to alfalfa, 13- and 9-HPO lyase from cucumber do not have the same pH optimum. Cucumber 13-HPO lyase has an optimal pH of 6.5, whereas the pH optimum of cucumber 9-HPO lyase is 8 [15]. The specific activities of alfalfa HPO lyase for both types of substrates slightly decrease during the first 8 days of germination and are highest in the 123 000×*g* pellet which contains the microsomes. Typical results are shown in Fig. 1.

To find out if alfalfa seedlings contain one enzyme that accepts both types of substrates, or two enzymes each specific for one positional isomer like cucumber [15], alfalfa HPO lyase was partially purified by ultrafiltration, gel filtration and anion exchange chromatography. A clear, colorless solution was obtained and the purification factors obtained with this method were typically around 10. With anion exchange chromatography HPO lyase was separated from lipoxygenase,

Table 1
Substrate specificity of HPO lyase in a crude extract of alfalfa

Substrate	Specific activity (U/mg) ± S.D.	Relative activity[a] (%) ± S.D.
13-HPOD	0.15 ± 0.008	90 ± 5
13-HPOT	0.17 ± 0.008	100 ± 5
9-HPOD	0.077 ± 0.008	46 ± 10
9-HPOT	0.086 ± 0.004	51 ± 5

Enzyme activities were determined with the assay described by Vick [21]. The data are mean values of three crude extracts.
[a]Activity compared to the activity with 13-HPOT as a substrate.

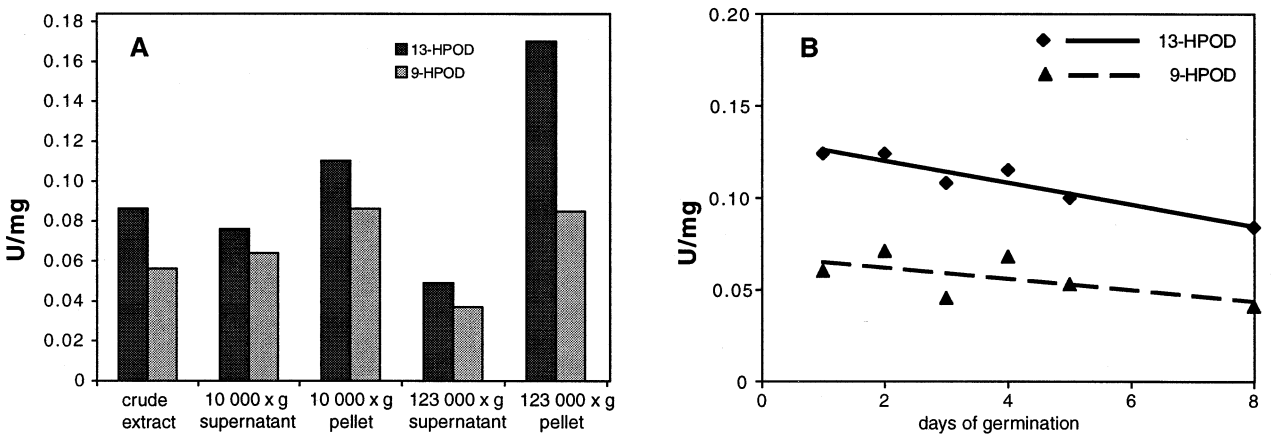

Fig. 1. HPO lyase activity in the different cell fractions of alfalfa seedlings (A) and during germination (B). HPO lyase activity was determined by measuring ΔA_{234} of a crude extract incubated with 13-HPOD and 9-HPOD. Crude extracts were prepared as described in Section 2.3 with 50 mM phosphate buffer pH 7.0, with (B) or without (A) 0.2% Triton X-100.

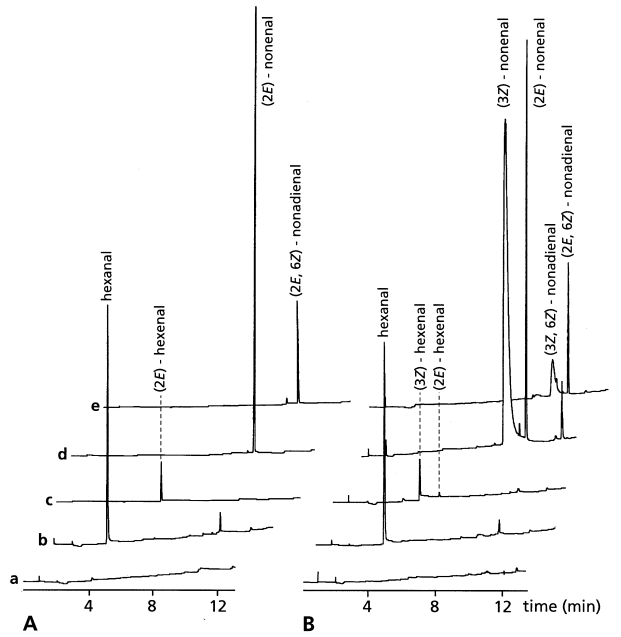

Fig. 2. Volatile reaction products formed by alfalfa HPO lyase obtained from the gel filtration pool (A) or from the anion exchange pool (B) incubated with (a) no substrate, (b) 13-HPOD, (c) 13-HPOT, (d) 9-HPOD and (e) 9-HPOT. Headspace compounds were trapped by SPME and analyzed by GC/FID. Identification of the compounds occurred by MS.

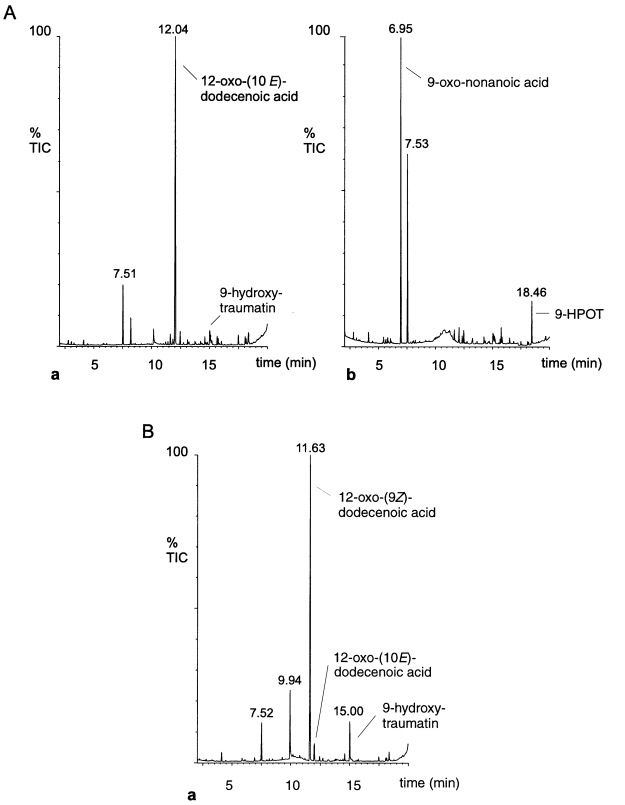

Fig. 3. Non-volatile reaction products formed by alfalfa HPO lyase obtained from the gel filtration pool (A) or from the anion exchange pool (B) incubated with (a) 13-HPOT and (b) 9-HPOT. GC/MS analysis of the methyl esters, trimethylsilyl ethers of the $NaBH_4$-reduced reaction products. The chromatograms of the incubations with 13-HPOD and 9-HPOD were similar to the chromatograms of the incubations with 13-HPOT and 9-HPOT, respectively.

but the 13- and 9-HPO lyase activities showed the same elution profile and could not be separated.

To obtain more information about the products formed by alfalfa HPO lyase, the following substrates were incubated with alfalfa HPO lyase obtained from the gel filtration pool: 13-HPOD, 9-HPOD, 13-HPOT and 9-HPOT. Headspace analysis of the incubation mixtures showed production of hexanal, (2*E*)-hexenal, (2*E*)-nonenal and (2*E*,6*Z*)-nonadienal, respectively (Fig. 2A). The absence of alcohols is probably caused by the loss of alcohol dehydrogenase during gel filtration, as headspace analysis of incubations of 13-HPOD and 13-HPOT with a crude extract of alfalfa did show production of hexanol and (2*E*)-hexenol. GC analysis of the non-volatile compounds in the incubation mixtures with 13-HPOD and 13-HPOT showed a large peak with a retention time of 12.04 min (Fig. 3A). Peaks of the following characteristic ions were present in the electron impact mass spectrum: *m/z* [ion attribution; relative intensity], 300 [M^+; 1.6%], 285 [M^+-CH_3; 8.7%], 253 [M^+-CH_3O_2; 51.9%], 129 [$C_3H_4OTMS^+$; 100%] and 73 [TMS^+; 81.1%], which corresponds to the mass spectrum of 12-oxo-(10*E*)-dodecenoic acid [6]. The mass spectrum of the peak at 15.00 min corresponds to 9-hydroxy-traumatin, another product of the lipoxygenase pathway recently described by Gardner [23]. GC analysis of the non-volatile com-

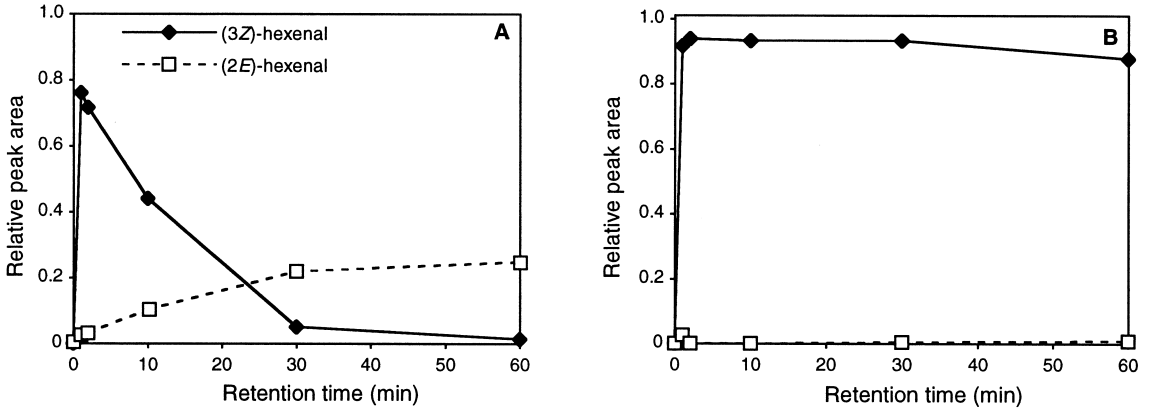

Fig. 4. Volatile products formed by a crude extract of alfalfa (A) and by partially purified HPO lyase (B) incubated with 13-HPOT. HPLC analysis after derivatization of the products with DNPH. The relative peak area is the ratio of product and internal standard peak areas.

pounds in the incubation mixtures with 9-HPOD and 9-HPOT showed a large peak with a retention time of 6.95 min. The electron impact mass spectrum showed peaks of the following characteristic ions: *m/z* [ion attribution; relative intensity], 245 [M^+-CH_3; 27.7%], 213 [M^+-CH_3O_2; 100%], 103 [CH_2OTMS^+; 34.2%], 89 [$OTMS^+$; 43.9%] and 73 [TMS^+; 71.6%]. This product was identified as 9-oxo-nonanoic acid. 13-HPOD and 13-HPOT were almost completely converted, whereas in the cases of 9-HPOD and 9-HPOT there was still substrate left. This might be due to the lower specific activity of alfalfa HPO lyase for the 9-isomers. The peak at 7.5 min also appeared in the sample without substrate and is therefore due to a component in the extract.

Remarkably, only the *E*-isomers were formed, which suggests the presence of an isomerase or an isomerization factor in alfalfa seedlings. When the same experiments were performed with further purified HPO lyase from the anion exchange pool, the *Z*-isoforms were the main products (Fig. 2B, Fig. 3B). The mass spectrum of the peak with retention time 11.63 min showed peaks of the following characteristic ions: *m/z* [ion attribution; relative intensity], 300 [M^+; 0.06%], 285 [M^+-CH_3; 3.2%], 253 [M^+-CH_3O_2; 11.6%], 103 [CH_2OTMS^+; 100%], 73 [TMS^+; 91.4%] and was identified as 12-oxo-(9*Z*)-dodecenoic acid. Thus, the isomerase or isomerization factor is lost during anion exchange chromatography. The loss of isomerase activity was also observed during the purification of cucumber HPO lyase [14]. To obtain more information about the 3*Z*:2*E*-enal isomerization, 13-HPOT was incubated for different periods of time with a crude extract or with HPO lyase obtained from the anion exchange pool. The products were derivatized with DNPH and analyzed with HPLC. As expected, the (3*Z*)-hexenal formed by a crude extract isomerized to (2*E*)-hexenal, whereas with the partially purified enzyme isomerization did not occur (Fig. 4). Remarkably, the isomerization appears to be a much slower process than the lyase reaction which was completed within 1 min. After boiling the crude extract for 5 min, no isomerization activity could be observed any more, which suggests involvement of an enzyme in the isomerization.

These results clearly show that both 13- and 9-HPO lyase activities and a 3*Z*:2*E*-enal isomerase are present in alfalfa seedlings. This makes an end to the former discrepancy between the product specificity of alfalfa lipoxygenase, which forms almost equal amounts of 13- and 9-hydroperoxy fatty acids [17,24], and the substrate specificity of HPO lyase, which was thought to use only the 13-isomers. The fact that alfalfa HPO lyase shows a high activity for both 13- and 9-substrates, and an almost equal activity for the hydroperoxides derived from linoleic or linolenic acid, makes it also very interesting as a biocatalyst for the production of short-chain aldehydes. The characterization of the enzymes and their application as biocatalysts are the subjects of future investigations.

References

[1] Major, R.T., Marchini, P. and Sproston, T. (1960) J. Biol. Chem. 235, 3298–3299.
[2] Zeringue, H.J. (1992) Phytochemistry 31, 2305–2308.
[3] Hatanaka, A., Kajiwara, T., Sekiya, J. and Toyota, H. (1986) Z. Naturforsch. 41c, 359–362.
[4] Gardner, H.W. and Plattner, R.D. (1984) Lipids 19, 294–299.
[5] Matsui, K., Shibutani, M., Hase, T. and Kajiwara, T. (1996) FEBS Lett. 394, 21–24.
[6] Vick, B.A. and Zimmerman, D.C. (1976) Plant Physiol. 57, 780–788.
[7] Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T. and Hatanaka, A. (1991) Phytochemistry 30, 2109–2113.
[8] Schreier, P. and Lorenz, G. (1982) Z. Naturforsch. 37c, 165–173.
[9] Shibata, Y., Matsui, K., Kajiwara, T. and Hatanaka, A. (1995) Plant Cell Physiol. 36, 147–156.
[10] Matoba, T., Hidaka, H., Kitamura, K., Kaizuma, N. and Kito, M. (1985) J. Agric. Food Chem. 33, 856–858.
[11] Fauconnier, M.-L., Perez, A.G., Sanz, C. and Marlier, M. (1997) J. Agric. Food Chem. 45, 4232–4236.
[12] Kim, I.-S. and Grosch, W. (1981) J. Agric. Food Chem. 29, 1220–1225.
[13] Gardner, H.W., Weisleder, D. and Plattner, R.D. (1991) Plant Physiol. 97, 1059–1072.
[14] Phillips, D.R. and Galliard, T. (1978) Phytochemistry 17, 355–358.
[15] Matsui, K., Shibata, Y., Kajiwara, T. and Hatanaka, A. (1989) Z. Naturforsch. 44c, 883–885.
[16] Sekiya, J. and Hatanaka, A. (1977) Plant Sci. Lett. 10, 165–169.
[17] Sekiya, J., Kajiwara, T. and Hatanaka, A. (1979) Agric. Biol. Chem. 43, 969–980.
[18] Chou, S.-R. (1991) Production of C6-aldehydes by Cultured Alfalfa Cells, PhD Thesis, Rutgers, The State University of New Jersey, New Brunswick, NJ.
[19] Elshof, M.B.W., Janssen, M., Veldink, G.A. and Vliegenthart, J.F.G. (1996) Rec. Trav. Chim. Pays-Bas 115, 499–504.
[20] Suurmeijer, C.N.S.P., Pérez-Gilabert, M., van der Hijden, H.T.W.M., Veldink, G.A. and Vliegenthart, J.F.G. (1998) Plant Physiol. Biochem. 36, 657–663.
[21] Vick, B.A. (1991) Lipids 26, 315–320.
[22] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[23] Gardner, H.W. (1998) Lipids 33, 745–749.
[24] Chang, C.C., Esselman, W.J. and Clagett, C.O. (1971) Lipids 6, 100–106.