Direct Release of Ethylene from Methionine and Methionine Containing Peptides by Action of 13-Hydroperoxy-(9*cis*, 11*trans*)-octadecadienoic Acid

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13-Hydroperoxy-9*cis*,12*trans*-octadecadienoic acid (13-LOOH) reacts with methionine and methionine-containing peptides in absence of any other reagent by slow release of ethylene. Ethylene was identified by mass spectrometry and quantified by gas chromatography. The amount of ethylene released in a certain time interval depends on the position of the methionine residue in the peptide chain. Highest rates of ethylene release were measured with peptides carrying methionine at the N-terminus. 13-Hydroperoxy-9*cis*,12*trans*-octadecadienoic acid can be substituted by linoleic acid, lipoxigenase and oxygen. We assume that the instant formation of lipid peroxides after plant injury and the instant release of 'wound ethylene' are related. Since the initiator tRNA in eucaryontic cells always carries a methionine, all newly produced proteins contain methionine at the N-terminal position and are therefore sensitive to oxidative damage by hydroperoxides of fatty acids.

Introduction

Ethylene, a volatile plant hormone, regulates ripening, cell growth and other physiological processes in plants (Burg, 1973). A strong increase of ethylene release is observed during both senescence (Guclu, 1989) and cell injury ('wound ethylene', (Kacperska, 1993)).

In addition ethylene is produced in the body during diseases which are connected with an increase in lipid peroxidation (Halliwell, 1987). Thus the determination of ethylene in exhaled breath is used as a non invasive method to determine the extend of oxidative cell injury.

The main source of plant ethylene is methionine: methionine is transformed by the enzyme ACC-synthase into 1-aminocyclopropane carboxylic acid. By action of ACC oxidase (ethylene forming enzyme, EFE) (Adams and Yang, 1979) ACC is cleaved in an aerobic reaction to form ethylene. ACC oxidase was isolated from apple tissue, purified and characterized (Dong, 1992). In addition, ethylene is liberated from ACC by radicals, e.g. by a system containing lipoxygenase, linoleic acid, Mn^{2+} and pyridoxal phosphate. This system produces hydroperoxy linoleic acid (Bousquet, 1984; Pirrung, 1986; Gardner, 1987) which is decomposed to radicals. Consequently ethylene production in microsomal membranes is enhanced in presence of hydroperoxy fatty acids (Lynch, 1985). Liberation of ethylene from ACC was also reported to be achieved with H_2O_2 and pyridoxal phosphate (Boller, 1979).

Another source of ethylene are ω-3 hydroperoxides derived from ω-3 fatty acids. If these hydroperoxides are cleaved in presence of Fe^{2+} ions ethylene is liberated.

In this communication we show that ethylene is liberated *in vitro* from methionine and methionine containing peptides by lipid hydroperoxides in absence of any other reagent. The rate in which ethylene is liberated depends from the location of the methionine in the peptide chain, as shown by the investigation of the following peptides: [5-methionine]-enkephalin (5-MET-ENK), N-formyl-methionyl-leucyl-phenylalanine (FMLP), glycyl-methio-

Abbreviations: ACC, 1-aminocyclopropane carboxylic acid; FID, flame ionization detector; FMLP, N-formyl-methionyl-leucyl-phenylalanine; GC, gas chromatography; GMG, glycyl-methionyl-glycine; HPLC, high pressure liquid chromatogaphy; ID, inner diameter; 13-LOOH, 13-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid; MET, methionine; 5-MET-ENK, [5-methionine]-enkephalin; MLP, methionyl-leucyl-phenylalanine; MS, mass spectrometry; PUFA, polyunsaturated fatty acid; rf, retention factor; rt, retention time.

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nyl-glycine (GMG) and methionyl-leucyl-phenylalanine (MLP).

Materials and Methods

GC

Head space measurements were carried out using a GSQ silica gel column (length: 30 m; inner diameter: 0.53 mm; carrier gas: H_2 2 ml/min; temp.-program: 60°C, 5 min, 60°-200°C at 10°C/min); detector: FID; injector-temp.:190°C.; detector-temp.: 210°C; split: 1:30; peak area integrator: Shimadzu C-R3A Chromatopac.

GC-calibration for ethylene

A given volume of pure ethylene (Linde) was deluted with argon in vials closed with a septum. The calibration curve was established by injection of given volumina of the gas mixture. Thus the curve represents the relation of ethylene amount versus peak area. This allowed the determination of the response factor for ethylene.

Preparation of 13-hydroperoxy-9cis,11trans-octadecadienoic acid

13-Hydroperoxy-(9,11)-octadecadienoic acid (13-LOOH) was prepared with soybean lipoxygenase (Fluka) and linoleic acid (Fluka) in presence of oxygen according to Teng (Teng *et al.* 1985). 13-LOOH was purified by TLC on 10x10 cm glass plates, coated with silica gel (Merck, Darmstadt) which were activated for two hours at 120° (100 mg/plate). The compounds in the crude mixture of oxidation products were separated with a 4:1 mixture of cyclohexane and ethyl acetate. The fraction between rf =0.2–0.4 (fluorescence at 254 nm) was scraped off and eluted with ethyl acetate. Most of the eluate was evaporated and subjected to a further purification by HPLC. HPLC was carried out with a C-18 column filled with 5 μm stearic octadecyl silica gel (ODS), (ID 8 mm, length 25 cm, flow 2.0 ml/min). The substances were eluted with a mixture of 98% H_2O, 2% acetonitrile (A) and 2% H_2O, 98% acetonitrile (B). Gradient: 0–100% B within 20 min, r_t of 13-LOOH: 21 min; detection: UV-detector at 212 nm.

The compound was identified by mass spectrometry after appropriate transformation (reduction, methylation, trimethylsilylation) with authentic material.

Oxidation of methionine containing peptides with 13-LOOH

1 μmol of methionine resp. 1-aminocyclopropane carboxylic acid resp. glycyl-methionyl-glycine resp. methionyl-leucyl-phenylalanine resp. N-formyl-methionyl-leucyl-phenylalanine resp. tyrosyl-glycyl-glycyl-phenylalanyl-methionine ([5-methionine]-enkephalin) was added to a phosphate buffer (pH 9.0) containing 1 μmol 13-LOOH. The reaction mixtures were filled into vials of 2 ml volume under an oxygen atmosphere. The vials were closed immediately with a septum and incubated in a bath of glycerol at 37°C.

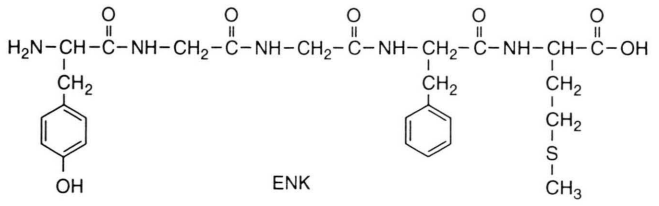
Determination of ethylene

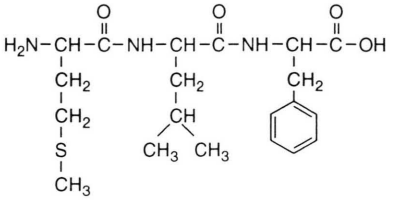
After the times indicated in table I a vial was removed. 500 μl gas samples were removed with a gas tight syringe (Hamilton) through the septum and immediately injected into the gas chromatograph. The peak area was integrated and the amount of ethylene was calculated with the aid of the calibration curve.

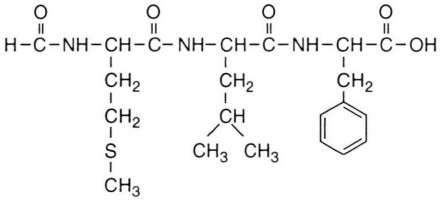
Results and Discussion

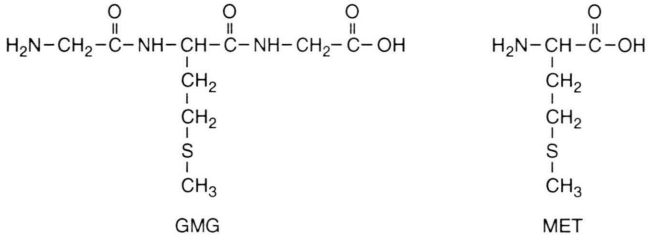
If methionine is treated with 13-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid (13-LOOH) ethylene is detected by mass spectrometry in the gas phase. Ethylene was quantified by gas chromatography after determination of the response factor by use of a calibration curve. Methionine does not occur in tissue in free form in higher concentrations. Therefore we investigated, if methionine containing peptides also produce ethylene in presence of 13-LOOH. [5-methionine]-enkephalin (5-MET-ENK), N-formyl-methionyl-leucyl-phenylalanine (FMLP), glycyl-methionyl-glycine (GMG) and methionyl-leucyl-phenylalanine (MLP) released different amounts of ethylene. Its production rate depends on the position of the methonine residue in the peptide chain (see Scheme 1).

The amount of ethylene which is released from methionine increases within 4 days only to 5% (13.7 pmol) of the amount of ethylene obtained from 1-aminocyclopropane carboxylic acid (ACC) (237.9 pmol) within the same time. This amount









Scheme 1. Investigated methionine containing peptides.

increases using peptides in which the amino group of methionine is connected to a carbonyl group: peptides carrying formyl methionine at the N-terminus (FMPL) or those in which the methionine residue is located within the chain (GMG) – as in most proteins - released 37,6 pmol resp. 40,0 pmol ethylene within 14 days. An about sevenfold increase in the release of ethylene (92,1 pmol) compared to methionine was observed in peptides with the methionine residue at the N-terminus. Such peptides are produced during the peptide synthesis. Since the initiator tRNA in eucaryontic cells always carries methionine, all new generated proteins have a methionine residue at the N-terminus. This residue is not important in most cases for the function of the protein and often removed shortly after synthesis of the protein. These primarily produced proteins with a methionine residue at the N-terminus obviously are especially exposed to oxidative damage by hydroperoxides of unsaturated fatty acids. A free carboxylic group in the methionine residue prevents the ethylene release: with e.g. 5-MET-ENK no release of ethylene was observed (see Fig. 1 and Table I).

The identified ethylene was not derived from 13-LOOH, because ethylene is generated exclusively by decomposition of ω-3 PUFAs. Hydroperoxides derived from ω-6 PUFAs, e.g. 13-LOOH, produce only pentane and 1-pentene (Scheick and Spiteller, 1995).

Ethylene formation by 13-LOOH has important physiological consequences in the hormone ho-

Table I. Formation of ethylene from 1 μmol of methionine (MET), methionyl-leucyl-phenylalanine (MLP), formyl-methionyl-leucyl-phenylalanine (FMLP), glycyl-methionyl-glycine (GMG), [5-methionine]-enkephalin (5-MET-ENK) and 1-aminocyclopropane carboxylic acid (ACC) by addition of 1 μmol 13-hydroperoxy-9*cis*,11*trans*-octadecadienoic acid.

Reaction time [d]	MET	MLP	FMLP	GMG	5-MET-ENK	ACC
2	2.3±0.89	–	–	–	–	81.1±3.23
4	3.8±1.17	–	–	–	–	141.9±26.85
7	7.7±1.04	40.5±4.3	14.4±1.43	16.8±2.28	0±0	216.5±30.12
9	8.8±1.04	–	–	–	–	232.1±25.71
11	10.4±1.19	–	–	–	–	252.3±16.02
14	13.7±1.93	92.1±5.51	37.6±4.03	40.8±5.55	0±0	273.9±21.61

meostasis of plants: Since ethylene stimulates the further release of ethylene from ACC (Buse *et al.*, 1993), the generation of ethylene from methionine containing peptides by action of 13-LOOH may have a control function. In addition destruction of methionine residues in peptides may change the tertiary structure of proteins to such an extend that enzymic functions are impaired. Membrane proteins could also be involved in such structural changes.

Injury of cells and the connected „oxidative stress" for the cells activates lipoxygenases (Capdevila *et al.*, 1990, Herold and Spiteller, 1995).

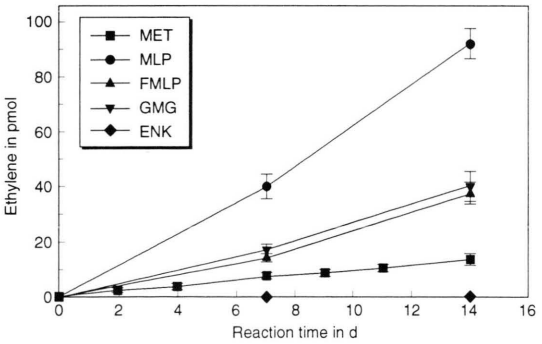


Fig. 1. Formation of ethylene from methionine containing peptides.

Thus the amount of hydroperoxides of fatty acids in these tissue increases and as a consequence an increased amount of ethylene should be liberated. Ethylene is oxidized *in vivo* by simultaneously activated epoxidases (Capdevila *et al.*, 1990) to ethylene epoxide which is a carcinogen (Beliles *et al.*, 1989) and mutagen (Basler, 1985).

Ethylene epoxide (Filser *et al.*, 1983) binds covalently and therefore irreversibly to histidine, lysine, valine and guanine residues of proteins and DNA (Segerbäck *et al.*, 1983, Törnqvist *et al.*, 1989) and causes as a consequence tissue injury.

In the peptides described in this investigation methionine was easily accessable and therefore exposed to oxidative attack by 13-LOOH due to the short peptide chains. In proteins methionine often is screened in the middle of the molecule and therefore protected. It was not investigated if such methionine residues are also damaged by attack of 13-LOOH.

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