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Assessment of lipoxygenase activity in seed extracts from 35 plant species

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RESUMEN

Evaluación de la actividad lipoxigenasa en extractos de semillas de 35 especies vegetales.

Se ha determinado la actividad lipoxigenasa en 35 extractos de semilla en base a la síntesis de hidroperóxido usando ácido linoleico como sustrato. Los resultados referidos al contenido en proteína extraídos mostraron que varias especies de *Vigna (V. unguiculata, V. radiata* y *V. mungo)* además de una de *Trifolium,* exhibieron mayor actividad lipoxigenasa que los extractos enzimáticos de soja. La relación entre la actividad-pH se ha establecido para 4 muestras muy activas. El análisis por GC-MS de estos extractos de semillas, mostró cantidades iguales de los hidroperóxidos 9- y 13 del ácido linoleico indicando la no especificidad posicional de la enzima.

PALABRAS-CLAVE: Extracto de semilla — Lipoxigenasa (actividad) — Vegetal.

SUMMARY

Assessment of lipoxygenase activity in seed extracts from 35 plant species.

Lipoxygenase activity in 35 seed extracts was determined on the basis of hydroperoxide synthesis using linoleic acid as substrate. The results referring to the extracted protein content show that several species of *Vigna (V. unguiculata, V. radiata* and *V. mungo)* and one of *Trifolium* exhibit stronger lipoxygenase activity than soybean enzyme extracts. The pH-activity relationship was also established for 4 very active samples. GC-MS analysis revealed equal amounts of 9- and 13- hydroperoxides of linoleic acid in these seed extracts, indicating no enzyme positional specificity.

KEY-WORDS: Lipoxygenase (activity) — Seed extract — Vegetable.

1. INTRODUCTION

Lipoxygenase (E.C. 1.13.11.12) has been reported to be ubiquitous in the plant kingdom (Gardner, 1991). Legume seeds are well known for their high lipoxygenase activity and that of soybean has been the more intensively studied (Siedow, 1991). The enzyme catalyses the addition of molecular oxygen to polyunsaturated fatty acids containing a *cis, cis*-1,4-pentadiene structure, e.g. linoleic or linolenic acid. It leads to the production of conjugated fatty acid hydroperoxides which are degraded to volatile aldehydes and alcohols and to ω-oxo fatty acids (Hamberg and Samuelsson, 1967).

Lipoxygenase-mediated oxydation of polyunsaturated fatty acids can be considered as a first step in the synthesis of flavouring compounds with typical "green notes". Depending on the origin of the enzyme, variable amounts of 9- and 13-hydroperoxides of, e.g., linoleic acid are formed (Chism, 1985). In biological systems, the hydroperoxides are cleaved by hydroperoxide lyases into aldehydes and ω-oxo fatty acids (Fig. 1) (Gardner, 1985; Croft et al., 1993); nevertheless, this reaction can also occur non-enzymatically by heating or by an acid-catalysed rearrangement (Gardner and Plattner, 1984). The aldehydes are often converted to their corresponding alcohols by alcohol dehydrogenases while *cis*-3-hexenal can be isomerized to the *trans* form by a *cis-trans* isomerase (Phillips, 1979). *Cis*-3-hexenol and *trans*-2-hexenal are known as "leaf alcohol and aldehyde" which are often added to flavouring compositions in order to enhance their "fresh note". *Cis, cis*-3,6-nonadienal has a typical pleasent cucumber flavour (Arctander, 1969). On the opposite, hexanal has a highly undesirable odor and causes problems for the preparation of food products containing soybean homogenates (Hildebrand, 1989 and 1984).

The present study is integrated within a research programme on enzyme-catalysed synthesis of flavouring compounds. The aim of this research is to find new efficient sources of vegetal lipoxygenases so as to product hydroperoxides which are the first step in the biosynthesis of green notes aldehydes. Seeds extracts presenting high lipoxygenase activities will be studied in detail afterwards to see if they can provide great amount of fatty acid hydroperoxides. We report herein on the measurements of lipoxygenase activity in seeds of 35 plant species belonging to different families. The relation between pH and activity is also established.

2. EXPERIMENTAL

Material

Linoleic acid (GLC purity > 95%) and Tween 40 were purchased from Sigma (St Louis, USA). Seeds were purchased from local market. All other reagents and solvents were of analytical grade.

Methods

The lipoxygenase assay was adapted from Surrey (Surrey, 1964).

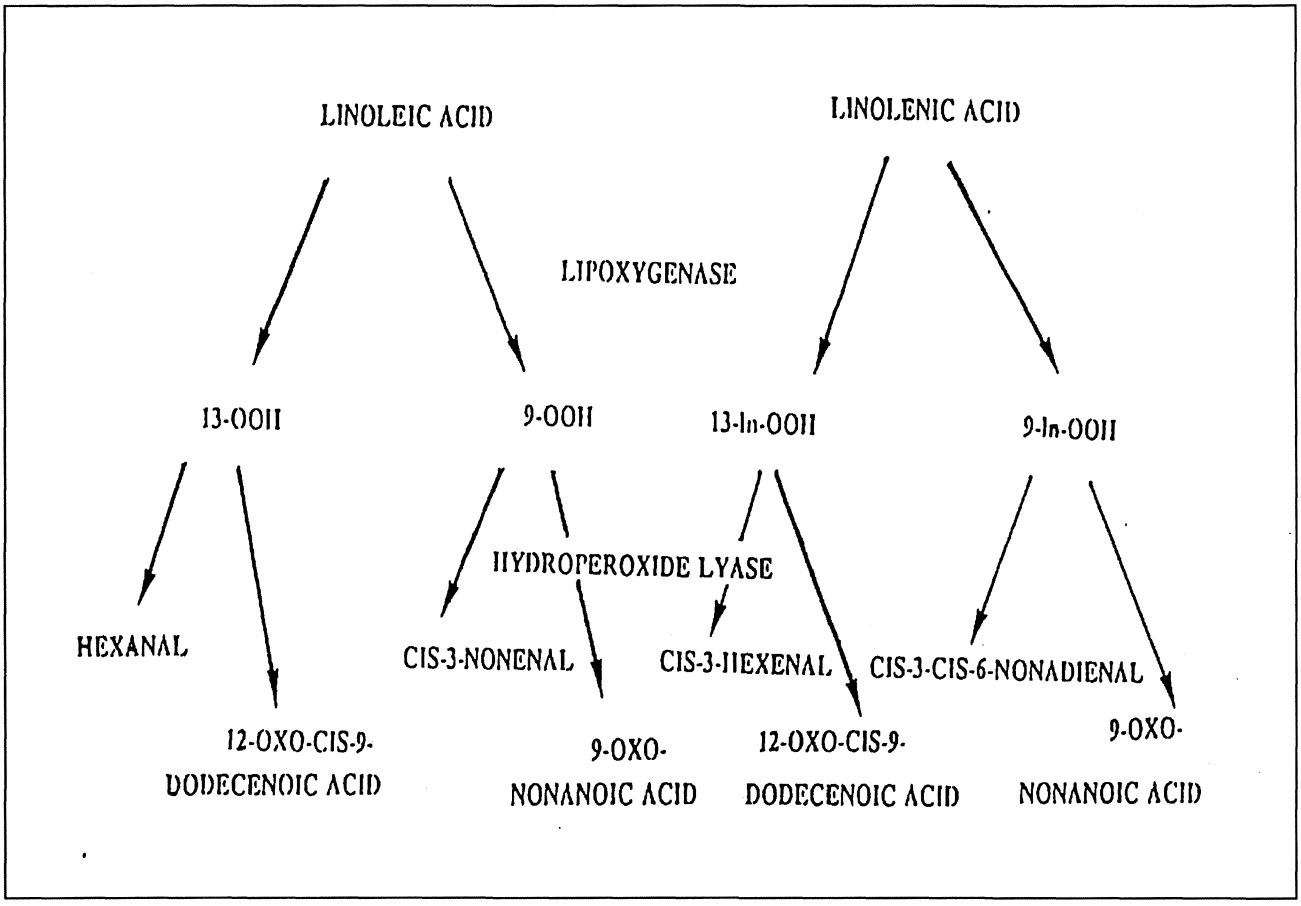

Figure 1
General scheme of lipoxygenase and hydroperoxide lyase activity on linoleic and linolenic acid (Vick and Zimmerman, 1993)

- Preparation of the emulsion: equal amounts (250 mg) of linoleic acid and Tween 40 were mixed. Five ml of a pH 9 borate buffer (0.02 M) and 0.65 ml of 1M NaOH were added under stirring. The emulsion was diluted with 45 ml of phosphate buffer (0,1 M) pH 7 and finally with water up to 100 ml. The pH was adjusted to 7.
- Preparation of the aqueous enzyme extract: one gram of ground seeds was extracted for 90 min at 0 °C with 100 ml of a pH 7 phosphate buffer (0.05 M). The extract was then centrifuged (3000 g, 10 min).
- Enzyme assay: 5 ml of the filtered extract were added to 100 ml substrate emulsion. The reactions were performed in narrow and long cylinders to avoid foaming. Pure oxygen was bubbled in the solution, the flow being adapted to insure O_2 saturation. After 2,4,8,15,30,45 minutes, 1 ml of sample was transferred into small centrifuge tubes containing 4 ml of absolute alcohol and then centrifuged at 3000 g for 5 min.
- Quantitative determination of linoleic acid hydroperoxides: the formation was estimated by measuring the increase of absorbance at 234 nm. All assays were done in triplicate. The results were expressed in µmol of hydroperoxides per gram of flour (dry weight) by second and in µmole of hydroperoxides per gram of protein in the extract by second.
- Protein content determination: total nitrogen content was determined both in flours and in enzyme extracts with a Kjeltec auto 1030 analyser from Tecator (Hoganas, Sweden) (protein = N x 6.25).
- The characterization of linoleic acid hydroperoxides was performed according to Hamberg and Samuelsson (Hamberg and Samuelsson, 1967). They were extracted with diethyl ether, reduced with $NaBH_4$ to the hydroxy-derivatives, hydrogenated in ethanol using Pt oxyde as catalist, converted to their methyl esters using methanol/ BF_3 and finally derivatized to trimethylsilyl ethers with BSTFA. The GC-MS analytical conditions were the following: SE 30 column (30 m X 0,25 mm i.d., d.f.: 0,25 µm) from Macherey-Nagel. Carrier gas: He (pressure of 0.8 bar). Temperature programme: from 60 °C to 240 °C at 6 °C / min and then at 20 °C / min until 270 °C. "Cold on-column"

injection. The mass spectra were recorded on a HP 5989 A spectrometer (70 eV, mass range scanned from 40 to 340 a.m.u.) coupled to a HP 5890 gas chromatograph (the characteristic fragments were respectively at m/z 173 and 315 for the derivative originating from 13-hydroperoxy linoleic acid as well as 229 and 259 for the derivative originating from 9-hydroperoxy linoleic acid corresponding to α-cleavage on each side of the hydroxy function).

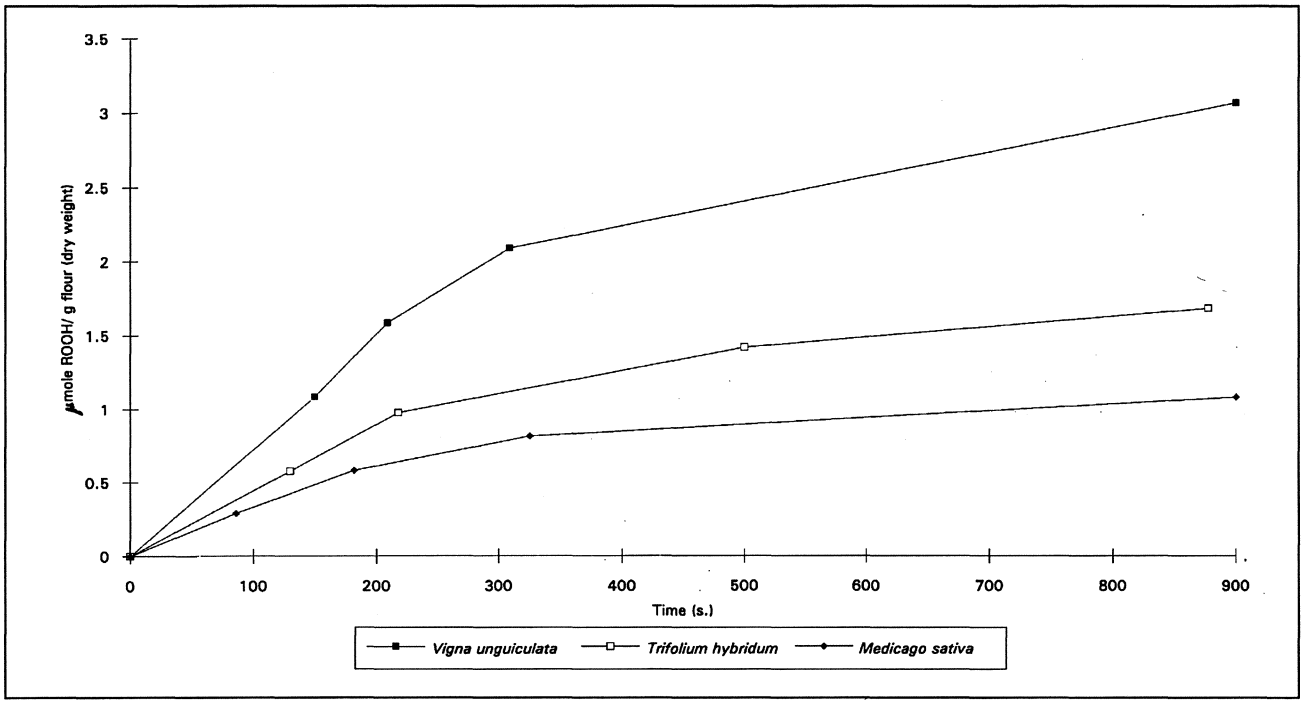

Figure 2
Production of linoleic acid hydroperoxides (ROOH) by enzyme extracts from seeds of *Medicago sativa, Trifolium hybridum* and *Vigna unguiculata* as function of time

3. RESULTS AND DISCUSSION

The initial part of the study was directed to the validation of the analytical procedures. For that purpose, the rate of hydroperoxide production was systematically determined over a period of 45 min for each sample tested. The enzymatic activities were determined in the part of the curve showing a linear relationship between reaction time and amount of product formed. Some typical examples are presented in figure 2.

The lipoxygenase activities of 35 aqueous seed extracts evaluated in terms of linoleic acid hydroperoxide synthesis are summarized in table I. Most of them had never been studied previously. The enzymatic activities were compared to that of *Glycine max* which was taken as a reference.

The examination of the data leads to the following observations:

- as suspected, a lot of species belonging to the *Fabaceae* exhibit a high lipoxygenase activity *(Glycine, Phaseolus, Trifolium, Vigna).*
- when the results are related to the protein content in the extract, several species of *Vigna* but also one of *Trifolium* show the highest level of biosynthetic capabilities. It could be attributed to the fact that these aqueous protein extracts contain greater proportions or more active lipoxygenases. Indeed, the yield of protein extraction of *V. radiata* and *V. unguiculata* seeds is comparable to that of soybean, but in the present analytical conditions, the linoleic acid conversion is more efficient with *Vigna* proteins.

The pH-lipoxygenase activity relationship was established for four legume seeds (the more active one on the per gram of flour production basis). Figure 3 indicates that the optimal pH value was very close to 6 for *V. radiata, V. unguiculata,* and *Phaseolus coccineus.* The production yield compared for each seed extract at their optimum pH shows that *V. radiata* is more efficient for hydroperoxide synthesis than *G. max* (15.5 and 11.4 µmole / g flour dry weight.s, respectively). Two optimal values were observed for soybean enzyme extract. They may correspond to isozyme 1 (optimum pH between 9 and 10) as well as isozymes 2 and 3 (optimum pH between 6 and 7) (Gardner, 1989). In the future, our investigations will focus on the study of *V.* lipoxygenases: purification, characterisation of activity, separation of eventual isozymes.

Table I
Lipoxygenase activity of different seed extracts reported to the dry weight and to the protein content in the extract. (ROOH: linoleic acid hydroperoxides)

	Dry weight (%)	Protein content in the extract (%)	μmole of ROOH /g flour (dry weight).s	μmole of ROOH /g protein (dry weight).s
Asteraceae				
Helianthus annuus	92.9	5.9	*	*
Brassicaceae				
Brassica napus	89.6	10.8	*	*
*Raphanus sativus***	94.4	11.9	*	*
Fabaceae				
Cicer arietinum	91.0	9.4	2.3 (±0.4)	24.8 (±4.8)
Glycine max	88.1	15.3	7.6 (±0.8)	49.4 (±5.2)
*Lens esculentum***	88.7	16.4	1.4 (±0.2)	8.5 (±1.2)
*Lotus corniculatus***	89.6	8.9	0.2 (±0.0)	2.6 (±0.5)
Lupinus alba	92.5	20.6	1.4 (±0.3)	6.7 (±1.2)
*Lupinus luteus***	90.1	26.1	0.4 (±0.1)	1.6 (±0.3)
*Lupinus polyphyllus***	89.9	14.8	3.4 (±0.4)	23.1 (±2.6)
*Medicago sativa***	91.2	15.5	2.9(±0.4)	18.7 (±2.4)
*Ocnitopus sativus***	91.4	5.3	*	*
Phaseolus coccineus	88.9	15.8	4.7 (±0.5)	29.5(±3.2)
Phaseolus vulgaris	90.2	13.3	3.7 (±0.6)	28.2 (4.2)
Pisum sativum	90.1	13.6	3.8 (±0.4)	28 (±3.3)
*Robinia pseudoacacia***	93.0	13.1	1.6 (±0.3)	12.6 (±2.2)
*Trifolium alexandrinum***	89.7	9.8	3.4 (±0.5)	34.4 (±4.8)
*Trifolium arvense***	90.5	13.6	3.6 (±0.5)	26.8 (±3.6)
*Trifolium hybridum***	93.5	6.4	4.6 (±0.8)	71.5 (±12.6)
*Trifolium incarnatum***	88.3	15.0	1.9 (±0.4)	12.8 (±2.5)
*Trifolium pratense***	91.9	9.4	3.7 (±0.7)	39.3 (±7.3)
*Vicia faba***	89.5	11.0	4.3 (±0.6)	39.2 (±5.3)
Vicia sativa	89.1	12.4	3.3 (±0.6)	26.3 (±5.0)
*Vigna angularis***	93.7	7.3	4.8 (±0.9)	65.7 (±12.1)
*Vigna mungo***	93.2	4.6	2.4 (±0.3)	51.4 (±7.0)
*Vigna radiata***	90.0	6.9	1.3 (±0.3)	19.5 (±2.2)
Vigna unguiculata	89.9	10.9	7.2 (±1.3)	65.8 (±11.5)
Linaceae				
Linum usitatissimun	94.0	11.9	0.9 (±0.1)	7.9 (±1.1)
Poaceae				
*Dactilys glomerata***	92.0	1.8	*	*
*Festuca pratensis***	90.7	2.3	*	*
*Lolium multiflorum***	91.2	2.2	*	*
*Lolium perenne***	89.7	2.3	0.2 (±0.0)	10.2 (±1.7)
*Phleum pratense***	89.8	1.8	*	*
*Poa trivialis***	91.3	1.2	*	*
Zea mays	89.8	1.4	*	*

* no lipoxygenase activity detected
** seed extracts studied for the first time

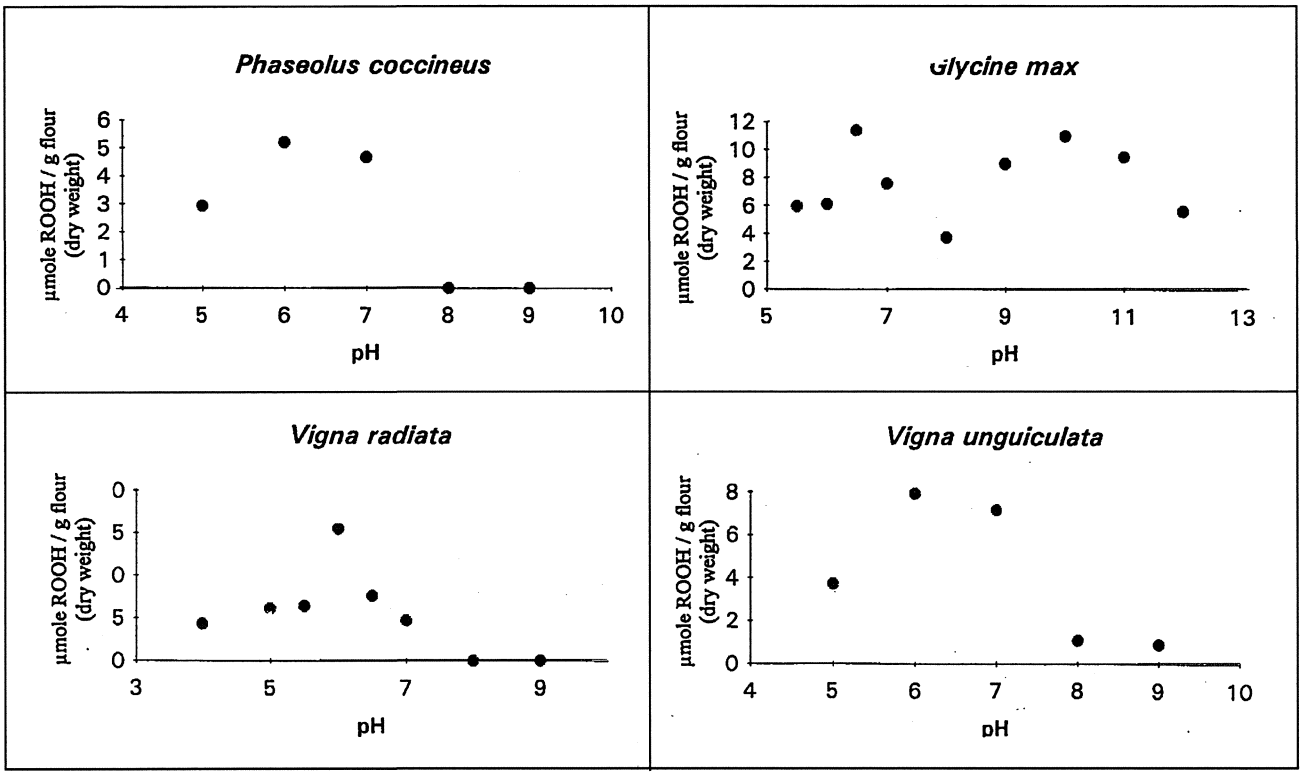

Figure 3
Relation between pH value and lipoxygenase activity for four legume seeds. (ROOH: linoleic acid hydroperoxides)

As reported in figure 1, two lipoxygenase-mediated pathways exist leading e.g. to 13- or 9-hydroperoxy linoleic acids. GC-MS investigations were undertaken in order to determine the enzyme specificity in four of the more efficient seed extracts *(G. max, V. unguiculata, V. radiata* and *P. coccineus).* At pH 7, equal amounts of 13- and 9-hydroperoxides were detected for all material tested, therefore the lipoxygenases were found to be non-specific. On the other hand, reactions carried out at pH 9 with soybean extracts show a strong lipoxygenase selectivity (80% of 13-hydroperoxy linoleic acid and 20% of 9-hydroperoxy linoleic acid). This is in line with the results of Axelrod (Axelrod, 1981).

In the future, we will focus our research on the study of *V.* lipoxygenases: purification, separation of eventual isoenzymes, characterisation of activity. The *V.* seed extracts will perhaps provide new sources of efficient vegetal lipoxygenases for the production of great amounts of fatty acid hydroperoxides.

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