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INHIBITION OF BROCCOLI LIPOXYGENASE BY SOME PHENOLIC COMPOUNDS – A SHORT REPORT

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Key words: lipoxygenase, LOX broccoli, phenolic compounds

The inhibitory effect of the selected phenolic compounds on the activity of lipoxygenase extracted from broccoli florets was analysed. Lipoxygenase was stable and demonstrated optimal activity at pH=6.5. Enzyme activity against linoleic acid was determined spectrophotometrically. Cinnamic acid, caffeic acid and n-propyl gallate proved to be uncompetitive inhibitors of broccoli lipoxygenase, whereas vanillin and benzoic acid – to be non-competitive inhibitors.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12, linoleate:oxygen oxidoreductase) is an iron-containing dioxygenase which catalyses the oxidation of polyunsaturated fatty acids and esters containing *cis*,*cis*-1,4-pentadiene system. Lipoxygenases (LOX) may also catalyse the co-oxidation of carotenoids, including β-carotene, resulting in the loss of essential nutrients and the development of off-flavours. The main reaction products are conjugated unsaturated fatty acids and hydroperoxides. In plants, lipoxygenase has been found in various organs and a comprehensive list of plant sources where it has been identified has been compiled by Whitaker [1991]. The physiological role of lipoxygenase in plants is not certain, although there is considerable evidence indicating its involvement in wounding and other stress responses [Rosahl, 1996]. Lipoxygenase activity is also the first step in the pathway leading to the formation of a number of flavour and aroma compounds [Barrett & Theerakulkait, 1995]. Lipoxygenases are of interest to food scientists because of their ability to form free radicals and peroxides which can be involved in indirect oxidation of carotenes and other food constituents. Various phenolics were commonly found in vegetables and some phenolic compounds are known to prevent lipoxygenase activity. Flavan and flavonols showed the highest inhibitory effects [Oszmiański & Lee, 1990] and the inhibitory potential of phenolic acids has not been well recognised so far [Richard-Forget *et al.*, 1995]. Better knowledge of the properties of lipoxygenase is essential to improve flavour stability of packaged food. Phenolic compounds have been repeatedly implicated as potent active antioxidants. These substances could act as scavengers for released free radicals or inhibit lipoxygenase activity which has been claimed to catalyse the oxidation of carotenoids and chlorophyll by a free radical mechanism.

The main objective of this investigation was to assess phenolic compounds as LOX inhibitors and to elucidate the mechanism of inhibition.

MATERIAL AND METHODS

Plant material. Commercial frozen broccoli florets (Hortex Company) – 100 g of material – were thoroughly ground in mortar with 100 mL of 50 mmol/L sodium phosphate buffer (pH=6.8) with 0.05% Triton X-100, and extracted in a magnetic stirrer for 1 h. The slurry was centrifuged (4000 g, 20 min).

The precipitate was discarded and the supernatant desalted with ammonium sulphate (VI) to 80% saturation. Enzymatic proteins were isolated and dialysed overnight against 0.01 mmol/L sodium phosphate buffer (pH=6.8). Dialysate was collected and centrifuged. The inactivated precipitate was discarded and the supernatant was fractionated with solid ammonium sulphate to 40% saturation. The precipitate was dissolved and dialysed overnight against 0.01 mmol/L buffer (pH=6.8) and collected in the final volume of 20 mL.

LOX activity. Lipoxygenase activity was determined spectrophotometrically by monitoring (Perkin-Elmer Lambda 5 uv-vis) the increase in absorbance at 234 nm [Theerakulkait & Barrett, 1995]. The standard assay mixture contained 0.3 mL of enzyme solution and 2.7 mL of substrate linoleic acid concentration 2.5 mmol/L in 0.1 mol/L sodium phosphate buffer at pH=7.0. One unit of enzyme activity was defined as an increase in absorbance of 0.001/min at 234 nm at 25°C.

Effect of pH on LOX activity. The reaction of the LOX enzyme was conducted in 3 mL of 0.1 mol/L sodium phos-

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phate (citrate buffers at pH=3–8) and initiated by the addition of linoleic acid Tween 20 emulsion at a final concentration of 2.5 mmol/L.

LOX inhibitors. The enzyme was preincubated for 10 min with inhibitors (vanillin, n-propyl gallate, benzoic acid, cinnamic acid, caffeic acid – from Sigma Aldrich) at a final concentration of 0.5 and 1.0 mmol/L, then the standard assays were conducted. Appropriate blanks and controls were performed through the same procedures.

RESULTS AND DISCUSSION

Enzyme recovery

The recovery of enzyme activity is dependent on the enzyme extraction method and assay conditions: acidity, the kind of substrate, and temperature. In the present study broccoli lipoxygenase was extracted with phosphate buffer at pH=6.8, following procedures described by Márczy *et al.* [1995]. These authors reported on slight differences in lipoxygenase activity depending on acidity and on the kind of buffer used to extract it from wheat germs. Only citrate buffer with pH=3.5 gave lower results, whereas in the case of acetate (pH=4.5 and 5.5) and phosphate (pH=7.0) buffer minimal differences were found in the activity.

This condition for extraction of lipoxygenase (pH=6.8) gave satisfactory extractability and there was no browning of the slurry which occurs when pH is higher than 7.1. To improve extractability, 0.01% of Triton X-100 was used. The higher concentration mostly (64%) decreased LOX activity and this may result from the replacement of natural lipids by detergents [Theerakulkait & Barrett, 1995]. The ammonium sulphate saturation of 40% was optimal and the recovery of activity was 61%.

Lipoxygenase activity accounted for 28 units/g of plant and was much higher than that reported by Zhuang *et al.* [1997], however, our assay was not standardised and similar units were defined as in the study of Theerakulkait and Barrett, [1995]. Linoleic acid is a substrate commonly used for determining lipoxygenase activity [Liagre *et al.*, 1996], however different concentrations of this acid have been recommended in literature: 106 μmol/L [López-Nicolás *et al.*, 2001], 1.4 mmol/L [Zhuang *et al.*, 1996], and 2.5 mmol/L [Theerakulkait & Barrett, 1995]. Determination of kinetic parameters for broccoli LOX indicated a relatively high K_m = 3.8 μmol/L compared to 1.4 μmol/L reported by Liagre *et al.* [1996] for eggplant, and thus confirms the recommendation of a higher substrate concentration to be used in LOX assays.

Isolation and purification

The precipitation of protein with ammonium sulphate is the most frequently used method at the first stage of enzyme purification. The subsequent two precipitations allowed easy separation of non enzymatic protein and other constituents from plant tissue. This method applied for the isolation of lipoxygenase from broccoli provided good results with the activity recovery of 61%. The crude enzyme dissolved in 0.05 sodium phosphate buffer was stable for 3–4 weeks at 2–8°C.

pH optimum

Figure 1 shows the pH optimum of LOX obtained with the use of linoleic acid. The maximum activity of linoleic acid occurred in a wide pH range, from 4 to 6.5. These values are different to those determined for many other lipoxygenases of LOX-pH activity. For lipoxygenase isolated from sunflower germs Leoni *et al.* [1985] found the activity within the pH range of 5.0–6.0 if the linoleic acid was the substrate, and an almost complete lack of changes depending on acidity when methyl linoleate was used as a substrate. In our trials the enzyme showed the maximum activity at pH ranging from 4.0 to 6.5 and its activity decreased rapidly in an alkaline medium. Such a wide optimum is unusual for the native enzyme, in this case it is rather the result of the analytical procedure, the protonation, and the aggregation of fatty acids. To avoid this problem, pH optimum should be determined using a method based on the formation of a complex between polyunsaturated fatty acids and cyclodextrin [López-Nicólas *et al.*, 1997].

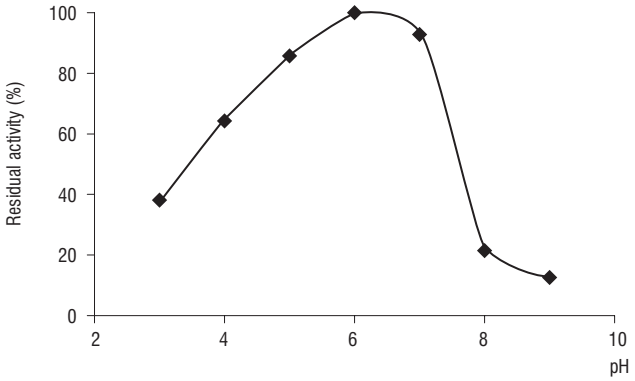

FIGURE 1. Effect of pH on broccoli lipoxygenase activity.

Inhibitory properties

The inhibitory properties of: benzoic acid, caffeic and cinnamic acid, vanillin and n-propyl gallate on broccoli lipoxygenase activity were compared using linoleic acid as a substrate. The inhibition constants Km_i were estimated from plots of apparent $1/V_m$ against inhibitor concentration (Table 1). All phenolic compounds exhibited affinity to a substrate-enzyme complex. Their apparent K_i values related with this affinity are in reciprocal relation to their antioxidative efficiency. Vanillin and benzoic acids were more potent inhibitors than caffeic and cinnamic acids. The type of inhibition was deduced from Lineweaver-Burk double reciprocal plots (Figures 2 and 3). Parallel lines were observed for the cinnamic and caffeic acids. These acids showed the nature of uncompetitive inhibitors. Vanillin and benzoic acid were non-competitive inhibitors. The strongest inhibitory activity was observed for vanillin. The present

TABLE 1. Inhibition constants of some phenolic compounds with broccoli lipoxygenase.

Phenolic compounds	K_i (mmol/L)
Benzoic acid	1.6
Vanillin	1.4
Cinnamic acid	2.6
Caffeic acid	1.8
n-Propyl gallate	1.6

study has confirmed the fact that the inhibitory ability depends on the structure and presence of hydroxyl groups. Caffeic acid, which is a 3,4-hydroxycinnamic acid, is a more potent inhibitor. Although in the studies of lipoxygenase isolated from seeds of *Pisum satuvum hortense* caffeic acid proved to be a slightly weaker inhibitor than the 3-hydroxycinnamic acid, it was more than two and four times stronger than 4-hydroxy- and 3,4-dihydroxyhydrocinnamic acids, respectively [Ligare *et al.*, 1996]. In the studies conducted by Richard-Forget *et al.* [1995], all the aromatic acids (hydroxyl derivatives of benzoic acid and their esters), derivatives of tannic acid and most of the tested flavonoids proved to be uncompetitive inhibitors of lipoxygenase isolated from horse bean, whereas flavanols (epicatechin, catechin, epigallocatechin) were uncompetitive inhibitors of both lipoxygenase isolated from horse bean and from barley germs. Only quercetin proved to be a non-competitive inhibitor in both cases. Gallate esters were also characterized as lipoxygenase inhibitors. In the present study, like in the quoted papers, n-propyl gallate showed an uncompetitive character of inhibition towards lipoxygenase isolated from broccoli. Propyl gallate strongly inhibited lipoxygenase linked with the soluble fraction of enzymatic proteins isolated from carnation petals, as well as lipoxygenase linked with the microsomal film of tomato [Rouet-Mayer *et al.*, 1992].

According to the recent reports a highly positive relationship between total phenols and antioxidant activity was found in many plant species [Vision *et al.*, 1998]. The edible parts of broccoli containing 56–71 mg/100 g of total phenols and 85% of phenolic fraction contain antioxidant agents. These concentrations are relatively low comparing to the values of inhibition constants in the present investigation, and the inhibition of lipoxygenase caused by phenolic compounds naturally occurring in plants should be also very low. Phenolic compounds which are endogenous inhibitors in plant sources could act mostly as scavengers of free radicals rather than as inhibitors of lipoxygenase generating free radicals released from fatty acid oxidation.

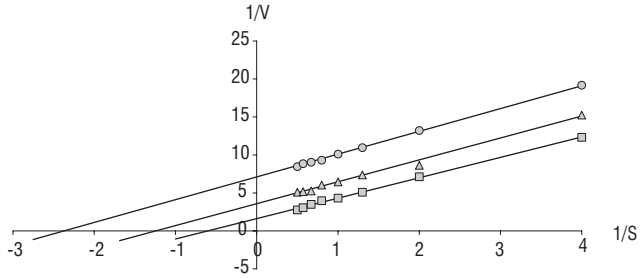

FIGURE 2. Inhibition of broccoli lipoxygenase activity by cinnamic acid.
○ – 1.0 mmol/L, △ – 0.5 mmol/L, □ – control

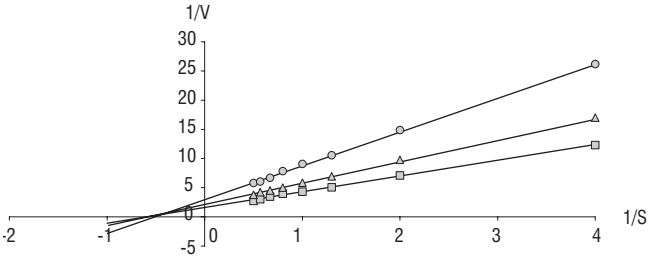

FIGURE 3. Inhibition of broccoli lipoxygenase activity by vanillin.
○ – 1.0 mmol/L, △ – 0.5 mmol/L, □ – control

CONCLUSIONS

1. The extract of broccoli florets exhibited the lipoxygenase activity in low acidity medium with the optimum at pH=6.5, whereas in an alkaline medium it was rapidly inactivated.

2. The phenolic compounds inhibited the activity of broccoli lipoxygenase. The mechanism of inhibition process was dependent on the molecular structure of phenolic compounds.

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INHIBICJA LIPOOKSYGENAZY Z BROKUŁÓW PRZEZ WYBRANE ZWIĄZKI FENOLOWE – KRÓTKI KOMUNIKAT

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Analizowano inhibicyjne działanie wybranych związków fenolowych na aktywność lipooksygenazy ekstrahowanej z brokułu. Aktywność lipooksygenazy oznaczano spektrofotometrycznie wobec kwasu linolowego. Kwas cynamonowy, kwas kawowy i ester propylowy kwasu tianinowego okazały się akompetycyjnymi inhibitorami lipooksygenazy brokułu, natomiast wanilina i kwas benzoesowy wykazały charakter inhibitorńw niekompetycyjnych (rys. 2 i 3). Wartości stałych inhibicji wyniosły od 1.4 mmol/L dla waniliny do 2.6 mmol/L dla kwasu cynamonowego (tab. 1). Wyizolowana z brokułu lipooksygenaza wykazała maksimum aktywności w pH=6.5, w środowisku alkalicznym ulegała gwałtownej inaktywacji (rys. 1).