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Purification and Characterization of Soluble *Cichorium intybus* Var. *silvestre* Lipoxygenase

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A water-soluble lipoxygenase enzyme (EC 1.13.11.12; LOX) occurring in the red cultivar produced in the geographical area of Chioggia (Italy) of *Cichorium intybus* var. *silvestre* was isolated and characterized. The molecular mass of the enzyme was estimated to be 74000 Da by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and gel filtration chromatography. The isoelectric point was pH 6.85. The optimum values of pH, ionic strength, and temperature, shown by isoresponse surface calculated by a randomized multilevel factorial design, were 7.58, 30 mM, and 38.5 °C, respectively. The enzyme showed high specificity toward linoleic acid, and the study of the variation of linoleic acid concentration between 30 and 300 μM, in the presence of Tween 20 at a concentration lower than the critical micelle concentration (0.01 v/v), resulted in a typical Michaelis−Mentem curve with K_M and V_{max} values of 1.49×10^{-4} M and 2.049 μM min^{-1} mg^{-1}, respectively. The biochemical properties, the kinetic parameters found, and the carotene-bleaching activity shown in aerobic conditions seem to indicate that the isolated enzyme is a lipoxygenase type III according to the indications given for soybean isoenzymes.

KEYWORDS: Red chicory; lipoxygenase; purification; biochemical characterization; kinetics

INTRODUCTION

Plant lipoxygenases (EC 1.13.11.12; LOX) are members of a class of non-heme iron-containing dioxygenases that catalyze the regio- and stereoselective oxidation of polyunsaturated fatty acids containing one or more 1(*Z*),4(*Z*)-pentadiene bond systems to produce (*E*,*Z*)-conjugated hydroperoxy fatty acids (*1*). LOX are widely distributed in mammals, plants, and fungi (*2*), whereas they are not, on the other hand, present in most bacteria, yeasts, and typical prokaryotes (*3*). A wide variety of plant LOX have been described and reviewed. LOX are ubiquitous in legumes, vegetables, and fruits (*4*) and have been isolated in many plants, particularly Leguminosae (*5*−*7*), Poaceae (*8*, *9*), Liliaceae (*10*), Solanaceae (*11*−*14*), Actinidiaceae (*15*), Oleaceae (*16*, *17*), and algae (*18*).

The biological role of plant LOX is not certain. The primary reaction products obtained from LOX activity, hydroperoxides, constitute the starting point for many reactions forming a variety of short-chain carbonyl compounds, which include aldehydes, ketone alcohols, acids, and epoxides, all of which are involved in vegetable growth, development, and senescence (*19*, *20*). Furthermore, LOX are involved in the synthesis of many compounds such as traumatin, abscissic acid, jasmonic acid hormone, and oxylipins, which are considered to play roles in the signal transduction during any wound response as well as for antimicrobial substances in host−pathogen interaction and, finally, and as a growth regulator (*21*−*28*).

The analysis of the functions of LOX in plants is complicated by the presence of multiple isoforms, which can occur with different tissue-specific expression levels within a plant. LOX isoforms can vary depending on developmental and environmental conditions (*1*, *29*). The existence of multiple lipoxygenase in soybeans is well-established (*1*), and the occurrence of different isoenzymes in other vegetables has been indicated.

Additionally, in fruits and other plant-food products, LOX are responsible for the production of a number of substances such as aromatic compounds implicated in the development of flavors and off-flavors (*30*, *31*). In some cases, LOX also contribute to the development of bitterness (*32*) and the change of color due to its pigment-bleaching ability (*33*).

In previous works, a number of vegetables commonly consumed in the Italian diet, considered to be traditional Italian food, belonging to the *Cichorium* genus, were studied to determine their anti- and pro-oxidant activities. In particular, the red cultivars of *Chicorium intybus* var. s*ilvestre*, which are grown in the winter season because of their resistance to cold temperatures, may be considered an important crop because of their availability throughout the entire year, providing an important source of nutrients during the cold season when fresh

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plants are far more scarce. The anti- and pro-oxidant activities of water-soluble components contained in red cultivars of *C. intybus* var. *silvestre* were investigated in three different chemical systems (*34*) and in two biological systems (*35*). The obtained results showed that despite the fact that the bitter red vegetables contain components with strong scavenger activity, they nevertheless showed prompt and strong pro-oxidant activity when tested with the linoleic acid−β-carotene assay, a method based on coupled oxidation of linoleic acid and β-carotene, that was used also to test secondary lipoxygenase activity (*36*).

The finding that among the chicory water-soluble components there are substances with carotene bleaching ability induced us to suppose the presence of a lipoxygenase.

As the plant LOX studied until today show a high heterogeneity in their biochemical characteristics (K_M, optimum pH, ionic strength, temperature, p*I*), a number of enzymatic forms and molecular masses, and as no studies on LOX in vegetables belonging to the Cichoriaceae family, in particular, *C. intybus* var. *silvestre* (red chicory), have been reported until now, as far as we know, we intend to confirm the presence of LOX in the red cultivar produced in the geographical area of Chioggia (Italy) of *C. intybus* var. *silvestre* (Chioggia red chicory) through its isolation and purification and to determine its biological properties and kinetic parameters.

MATERIALS AND METHODS

Vegetable Sample. The red cultivar produced in the geographical area of Chioggia (Italy) of *C. intybus* var. *silvestre* was purchased in a local supermarket during the winter months.

Preparation of the Crude Enzyme Extract (cLOXe). Forty grams of Chioggia red chicory leaves was homogenized in an ice bath in an Ultra Turrax containing 80 mL of phosphate buffer (0.06 M), at pH 6.6 for 1 min. The homogenate was shaken for 20 min at 4 °C to facilitate enzyme extraction. The mixture was then centrifuged at 5000 rpm for 20 min, then filtered through a Ruudfilter Schleicher Schuell 1573, and afterward filtered through Millipore membranes of cellulose acetate/cellulose nitrate mixed esters (0.45 μm).

Dialysis. Dialysis was performed in a Spectra/Por Biotech cellulose ester membrane with a molecular mass cutoff of 300 000 Da. A 9 mL aliquot of cLOXe was fractionated by dialysis in 900 mL of distilled water at 4 °C. Dialysis was performed for 3, 6, and 24 h. The retentates and dialysates were brought to the corresponding volume of cLOXe and then tested for pro-oxidant activity by the carotene bleaching activity assay.

Purification of LOX. The gel filtration chromatography (GFC) apparatus was a 655A-11 Merck-Hitachi liquid chromatograph with a variable-wavelength UV monitor at 280 nm. The GFC separation of the cLOXe or of dialysates (molecular mass < 300 000 Da) obtained after 6 and 24 h was performed using a Superformance Universal glass-cartridge system (300 × 10 mm) (Merck). The stationary phase was a TSK gel Toyopearl HW-55 (F) (exclusion limit 1000−150 000 Da) (Tosoh Biosep GmbH), and the mobile phase was Millipore grade water at flow rate of 0.5 mL min^{-1}. The injected volume was 1 mL, corresponding to 1 mL of cLOXe.

Determination of Protein Content. GFC fraction protein content was determined according to the method reported by Lowry et al. using bovine albumin as the standard protein (*37*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The molecular weight of the enzyme was determined by SDS-PAGE with a Multiphor II system and ExcelGel SDS homogeneous 7.5 (7.5% PAGE, separation range of 20−300 kDa). The enzyme molecular mass was calculated with a broad-range protein molecular weight marker (molecular mass standard range of 220−53 kDa) (Amersham Biosciences). About 5−15 μg of total protein was loaded per lane. Proteins were visualized by the Coomassie staining method (*13*).

Isoelectric Focusing (IEF). The isoelectric point of the enzyme was determined by electrophoresis with a Multiphor II system and an Ampholine PAGplate (pH range of 3.5−9.5). The enzyme isoelectric point was calculated with broad p*I* markers (p*I* standard range of 3.5−9.5). Proteins were visualized by the Coomassie staining method (*13*).

Conjugated Diene Assay. Lipoxygenase activity, based on the absorption at 234 nm of the conjugated dienes formed when linoleic acid used as substrate oxidized in the presence of the enzyme, was assayed according to the method of Chen and Whitaker (*38*) with some particular modifications. The stock substrate solution was prepared by mixing 19 μL of linoleic acid (Merck), 20 μL of Tween 20 (0.01% v/v) (Merck), and 5 mL of distilled water, clarified by adding 0.5 mL of 1 M NaOH. Before the assay, 110 μL of the stock solution was diluted with Tris-HCl buffer or phosphate buffer at different pH values (from 4.0 to 10.0) and ionic strengths (from 10 to 120 mM) to the final volume of 4 mL.

The reaction was initiated by the addition to 540 μL of substrate solution of 60 μL of enzyme, and the formation of hydroperoxide was followed for 10 min with the increase in absorbance at 234 nm. The control sample microcuvette contained 540 μL of substrate solution and 60 μL of distilled water, and the formation of hydroperoxide not due to the enzyme addition was followed for 10 min as reported for the sample. Each sample was read against a solution prepared as a substrate solution, but without linoleic acid. The lipoxygenase activity expressed as katals (moles of substrate per second) was calculated from the linear part of the curve (*11*). Specific lipoxygenase activity was espressed as katals per milligram of protein.

Carbonyl-Producing Activity Assay. The carbonyl-producing activity of lipoxygenase enzyme was determined spectrophotometrically by measuring the increase of absorbance at 280 nm. The reaction conditions were the same as for the conjugated diene assay (*39*).

Carotene Bleaching Activity Assay. The lipoxygenase activity of the cLOXe, all of the dialysates and retentates obtained at different times, and their GFC fractions, based on coupled oxidation of linoleic acid and β-carotene, was evaluated following the Taga method et al. (*40*) with some modifications (*41*). β-Carotene (5 mg) (Merck) was dissolved in 50 mL of chloroform solution. A 3 mL aliquot of β-carotene chloroform solution was added to a conical flask along with 40 mg of linoleic acid and 400 mg of Tween 20. Chloroform was evaporated, until dry, under reduced pressure at a low temperature (<30 °C). Distilled water (100 mL) was added to the dried mixture, and the mixture was then shaken. An aliquot (400 μL) of distilled water (control sample) or sample was added to 5 mL of β-carotene emulsion in a cuvette, and the mixtures were mixed well. The absorbance at 470 nm was measured immediately and then measured once again after 30 s for 10 min at room temperature. Each sample was read against an emulsion prepared as described but without β-carotene (blank). To correct for the influence of the juice color in the calculation of the β-carotene degradation percentage, an aliquot (400 μL) of each sample was added to 5 mL of blank (blank samples). These mixtures for each time point were spectrophotometrically read, and the absorbance measured was subtracted from that of the corresponding sample. The lipoxygenase activity was expressed either for the sample or for the control sample as relative β-carotene degradation percentage using the equation

$$\text{relative } \beta\text{-carotene degradation percentage} = 100 - (A_t/A_0) \times 100$$

where A_0 = absorbance of the sample (or control sample) − absorbance of blank sample (or control sample) at time 0 (absorbance read immediately after the addition of the cLOXe or dialysates and retentates) and A_t = absorbance of the sample (or control sample) − absorbance of blank sample (or control sample) at time *t*.

Effect of pH, Ionic Strength, and Temperature. The enzymatic activity was determined with the conjugated diene assay, using linoleic acid as a substrate. To determine the pH optimum, the enzyme activity was assayed in the range pH 4.0−10.0 at room temperature (20 °C). The buffer systems were 50 mM citrate phosphate buffer for pH 4.0 and 5.0, 50 mM sodium phosphate buffer for pH 6.0, 50 mM Tris-HCl buffer for pH 7.0−9.0, and sodium hydroxide−sodium borate buffer for pH 10. To determine the ionic strength optimum, the enzyme activity, expressed as millimolar, was determined in the range of ionic strength from 10 to 120 mM at room temperature, using Tris-HCl buffer

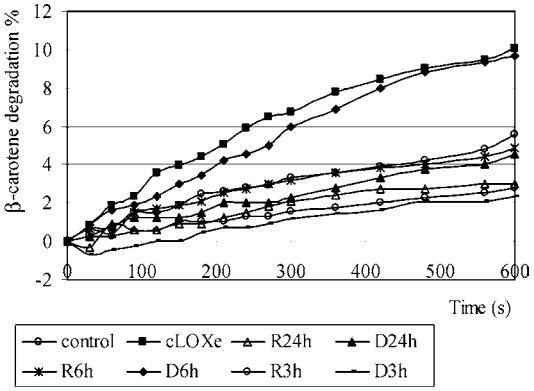

Figure 1. β-Carotene degradation percentage in the presence of crude enzyme extract, dialysates, and retentates obtained after 3, 6, and 24 h. Values represent means of five replications and SD values <0.3 β-carotene degradation percentage.

(pH 8.0). To determine the temperature optimum, LOX activity was determined within the range of 2−55 °C using Tris-HCl buffer (pH 8.0, 25 mM).

Statistical Analysis. The reported values represent means of at least five replications.

A randomized multilevel factorial design was used to plan the experimental conditions of the experiments by the statistical package StatGraphics Plus (4.1 version, 1999).

The enzymatic activity was considered as response variable and pH, ionic strength, and temperature as classification factors. Each factor was considered at three levels (high, medium, and low): pH, 7.0, 7.5, and 8.0; ionic strength, 20, 30, and 40 mM; temperature, 35, 40, and 45 °C), and the number of planned experiments was 27. The experimental design listed the experiments that obtained a response surface in order to determine, with the highest accuracy, the pH, ionic strength, and temperature values corresponding to the highest enzymatic activity.

The obtained data were analyzed through the analysis of variance (ANOVA) (confidence level of 95%).

Substrate Specificity. The enzyme activity toward linoleic acid, α- and γ- linolenic acid, trilinolein, and 1,3-dilinolein at 300 μM concentrations was measured under standard conditions (pH 7.58, ionic strength = 30 mM, and T = 38.5 °C) using the conjugated diene assay.

Effect of Substrate Concentration and Determination of Kinetic Parameters (K_M and V_{max}). The enzymatic activity was evaluated toward linoleic acid in the concentration range from 30 to 1200 μM. The kinetic constants of the enzyme were measured under standard conditions using linoleic acid concentrations ranging from 30 to 300 μM. The enzyme volume was always equal to 100 μL/mL of reaction mixture. All of the determinations were repeated three times, and the kinetic parameters were evaluated by plotting the data on the double-reciprocal graph.

RESULTS AND DISCUSSION

The vegetable samples belonging to the Chioggia red chicory underwent homogenization and extraction to isolate the water-soluble vegetable components suspected of containing the enzyme. The crude LOX extract was tested for lipoxygenase activity. As the use of the conjugated diene method with crude enzyme preparations is limited by the interference of other UV-absorbing material, as reported by Ben-Aziz et al. (*42*) and by Anthon and Barrett (*43*), the the lipoxygenase activity of the cLOXe was determined only with the method based on the lipoxygenase secondary reaction that causes the carotene bleaching. The results reported in **Figure 1** show that the β-carotene degradation percentage was higher in the presence of cLOXe in comparison to the control sample after only 30 s

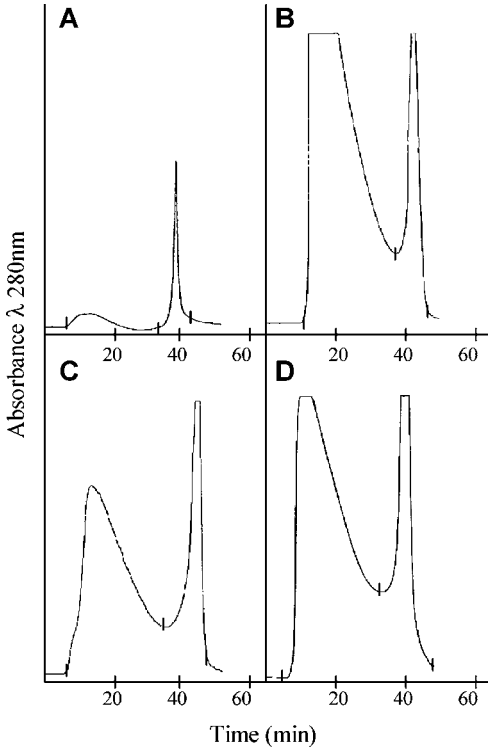

Figure 2. (**A**) GFC of standard lipoxygenase; (**B**) GFC of crude enzyme extract; (**C**) GFC of dialysate obtained after 6 h; (**D**) GFC of dialysate obtained after 24 h. Operative conditions are described in the text.

of reaction, demonstrating that there was no induction period in the lipid oxidation, as when a lipoxygenase actually is in action.

To isolate the enzyme probably occurring in the crude extract and to have a preliminary indication about its molecular mass, the cLOXe was submitted to dialysis (cutoff 300 000 Da) for 3, 6, and 24 h, respectively. Also, the retentates and the dialysates, due to the presence of interfering compounds, could be tested only with the method based on carotene bleaching ability to determine their enzymatic activity. The results, reported in **Figure 1**, show that the highest β-carotene degradation percentages were given by the dialysate obtained after 6 h of dialysis (D6h), which showed an enzymatic activity close to that of the cLOXe. Conversely, β-carotene degradation percentages, obtained from all of the retentates (R24h, R6h, and R3h), were lower and close to the values given by the dialysates obtained after 3 h (D3h) and after 24 h (D24h) of dialysis, indicating that 3 h is not long enough to completely recover the lipoxygenase enzyme, whereas 24 h is too long a period and produces a decrease in the carotene bleaching ability of the dialysate components.

To purify LOX, a GFC preparative was applied, using blue dextran to determine the dead volume time and a buffered solution of soybean LOX (Sigma) as a standard compound. The eluate obtained from standard soybean lipoxygenase monitored at 280 nm (**Figure 2A**) showed two peaks, the first of which had a retention time of 15.6 ± 0.9 min and the second one a retention time of 39.5 ± 0.5 min. The GFC eluates corresponding to the two chromatographic peaks, GFC1 and GFC2, restored to the initial volume of the standard enzyme solution, both showed lipoxygenase activity, which could be determined with either the conjugated diene assay or the carotene bleaching activity assay. This suggested that the first fraction, the less

Table 1. Secondary Enzymatic Activity: Carotene Bleaching Activity of LOX Crude Extract, Dialysates Obtained after 6 and 24 h, and Their GFC Fractions

sample	linoleic acid–β carotene assay (relative β-carotene degradation, %)
cLOXe	3.60
GFC1ce	1.88
GFC2ce	4.70
D6h	3.37
GFC1D6h	1.54
GFC2D6h	2.72
D24h	1.28
GFC1D24h	1.52
GFC2D24h	1.85

Table 2. Primary Enzymatic Activity: Hydroperoxide Formation Activity of GFC Fractions Prepared from Crude Enzyme Extract and Dialysates Obtained after 6 and 24 h

sample	protein (mg/mL)	LOX activity (nkat)	specific activity (nkat/mg)
GFC1ce	1.127	0.75	0.66
GFC2ce	0.488	1.20	2.46
GFC1D6h	0.852	0.50	0.59
GFC2D6h	0.462	1.18	2.55
GFC1D24h	0.912	0.76	0.83
GFC2D24h	0.467	1.21	2.59

active, which had the same retention time as blue dextran, was probably a protein dimero occurring in the standard soybean LOX.

The crude LOX extracts of the tested vegetable and the dialysates obtained from it after 6 and 24 h of dialysis, respectively, were also analyzed by GFC. All gave two chromatographic peaks, as the standard soybean LOX (**Figure 2B−D**). The first peak had the same retention time as blue dextran and the second one a retention time (39.7 ± 0.5 min) close to that of the standard soybean LOX. The two GFC fractions obtained, either from the cLOXe or from the two dialysates (6 and 24 h) restored to the initial volume of the enzyme extract, could be tested for LOX activity either with the carotene bleaching activity assay or with the conjugated diene assay. Moreover, as it is known that some lipoxygenases possess, besides the above-reported ability in carotene bleaching, even another secondary enzymatic activity, that is, the ability to catalyze the hydroperoxide degradation into keto-diene acids (*39, 44*), all GFC fractions were tested also for this secondary enzymatic activity with the carbonyl-producing activity assay.

When GFC fractions were tested for carotene bleaching ability, all GFC1 fractions showed low activity. With regard to the GFC2 fractions, the results showed that the GFC2 fraction obtained directly from the crude extract had the higher β-carotene bleaching activity, whereas that obtained after dialysis of the crude extract was less active, with lipoxygenase activity that decreased with the increase of dialysis time (**Table 1**). These results might indicate that the enzyme lost its secondary enzymatic activity or, as the pigment bleaching ability is related to the formation of hydroperoxide, they might also indicate that the enzyme lost its primary enzymatic activity with the increase of dialysis time.

The same fractions when tested with the conjugated diene assay to determine the primary lipoxygenase activity gave different results (**Table 2**). In fact, all of the GFC2 fractions had high lipoxygenase activity, with similar values, independent of dialysis time. These results were confirmed by the specific

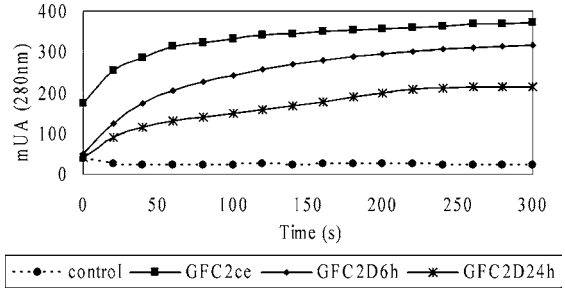

Figure 3. Secondary lipoxygenase activity of *C. intybus* LOX: formation of carbonyl compounds. Values represent means of three replications and SD values <4 mUA.

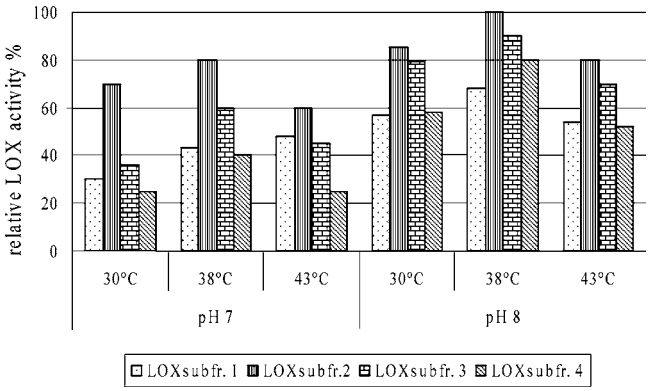

Figure 4. Lipoxygenase activity of GFC2 subfractions at different temperatures and pH values.

activity values, which are similar for all three GFC2 fractions. With regard to the GFC1 fractions, the diene assay showed that they had low activity.

These findings show that the dialysis process affects the secondary enzymatic activity only.

When GFC fractions were tested for the ability to catalyze the hydroperoxide degradation into keto-diene acids, the GFC2 fraction obtained directly from crude enzyme extract showed a remarkably higher activity than GFC2 fractions obtained after dialysis (**Figure 3**). These results showed that the dialysis process, which does not influence the primary enzymatic activity, conversely, seriously affected also this secondary enzymatic activity, that is, the hydroperoxide degradation.

We can suppose that the variations found in the GFC2 fraction secondary enzymatic activities that become more and more significant as the dialysis time lengthens might be due to modification of LOX protein during dialysis purification, which does not involve the catalytic domain responsible for the substrate binding.

To determine if the considered GFC2 fraction contains more than one LOX enzyme form, the corresponding GFC eluate was further separated into four subfractions, which were analyzed with diene assay in different operative conditions. The subfractions showed, in preliminary tests at different temperatures and pH values, the same biochemical characteristics but different enzymatic activity values, with the highest activity corresponding to GFC2 subfraction 2 (**Figure 4**). These results indicated that all subfractions contain the same enzymatic form even if in different concentrations, as we expected.

The presence of just a LOX isoform in the GFC2 fraction was confirmed by the analytical isoelectric focusing (**Figure 5A**) and SDS-PAGE analysis (**Figure 5B**). In fact, both analyses showed a single electrophoretic band with an isoelectric point of 6.85 and a molecular mass of 74000 Da, respectively.

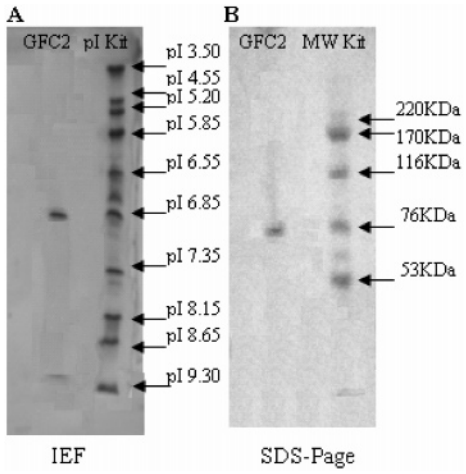

Figure 5. (**A**) IEF analysis of GFC2 *C. intybus* LOX; (**B**) SDS-PAGE analysis of the GFC2 *C. intybus* LOX. Ip st, isoelectric point standards; MW st, molecular weight standards.

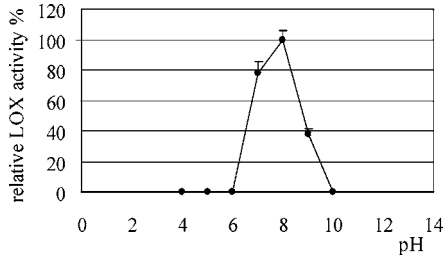

Figure 6. Effect of pH on *C. intybus* LOX activity with linoleic acid (300 μM) as substrate in Tris buffer (50 mM) at room temperature. Values represent means of five replications.

The most active GFC2 subfraction 2 was used to determine the most important biological properties (pH, ionic strength, temperature, and substrate specificity) and the kinetic parameters.

The enzyme pH optimum was tested using linoleic acid as a substrate in a pH range from 4.0 to 10.0. LOX activity showed its maximum at pH ~7.5 within a 50 mM Tris-HCl buffer, but the activity was also appreciable within the range from pH 7.0 to 9.0, showing that the enzyme activity is more sensitive to an acid than to an alkali medium (**Figure 6**). A similar pH profile is reported for LOX III, found in a number of vegetables (*14*, *16*).

The effect of ionic strength on LOX activity was investigated at the optimum pH value found previously (pH 8). Six different concentrations of Tris-HCl buffer were tested, and LOX activity showed its maximum at a buffer concentration of ~25 mM. At lower and higher buffer concentrations the activity rapidly decreased until, at a buffer concentration of 10 mM, LOX activity was ~20% of its initial activity (**Figure 7**). Similar values of ionic strength optimum for the enzymatic activity were found in another study on kiwi fruit LOX, where the high ionic concentration (>0.2 M) inhibited LOX activity (*15*).

The effects of temperature on *C. intybus* LOX activity were evaluated in the range between 2 and 55 °C. The enzyme activity increased with the rise of temperature from 2 to 35 °C and then declined rapidly (**Figure 8**). The optimum temperature appeared to be ~35 °C. Similar temperature ranges are reported for other vegetables, where the optimum temperature was indicated in the range from 30 to 40 °C (*8*, *15*, *17*).

To define the exact values of the considered biological parameters, a randomized multilevel factorial design was used.

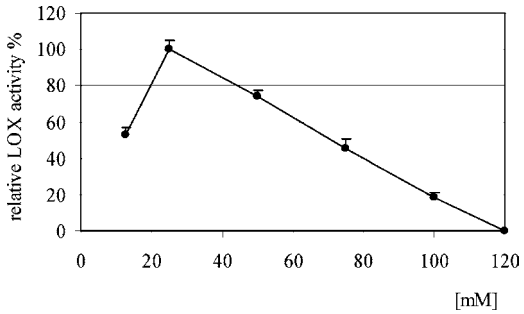

Figure 7. Effect of ionic strenght on *C. intybus* LOX activity with linoleic acid (300 μM) as substrate in Tris buffer (pH 8.0) at room temperature. Values represent means of five replications.

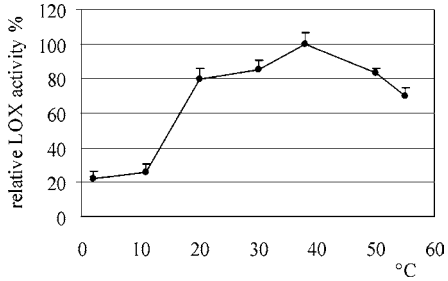

Figure 8. Effect of temperature on *C. intybus* LOX activity with linoleic acid (300 μM) as substrate in Tris buffer (pH 8.0–25 mM) at room temperature. Values represent means of five replications.

Table 3. Substrate Specificity of *C. intybus* LOX

substrate	relative LOX activity (%)
linoleic acid	100.00
α-linolenic acid	42.98
γ-linolenic acid	49.50
1,3-dilinolein	0.00
trilinolein	15.50

The enzymatic activity was considered as a response variable, and pH, ionic strength, and temperature were considered to be classification factors. The high, medium, and low levels around the preliminarily determined optimum values were established for each classification factor. The experimental design listed the experiments that permitted determination with the highest accuracy of the pH, ionic strength, and temperature values corresponding to the highest enzymatic activity.

The analysis of variance (ANOVA) showed that enzymatic activity is significantly different ($p < 0.05$) across the three different values of applied temperature, whereas the three different values of pH and ionic strength do not significantly affect the enzymatic activity. Furthermore, ANOVA indicated that there is an interaction between the temperature and ionic strength factors ($p = 0.0339$), whereas no interactions between the other couples of factors were found. Therefore, the temperature and ionic strengths were found to be not independent factors.

The optimum values of pH and temperature shown by isoresponse surface were pH 7.58 and $T = 38.5$ °C, respectively, for a 30 mM ionic strength value.

The relative enzyme activities toward various substrates, that is, polyunsaturated fatty acids and acylglycerols, were determined at a substrate concentration of 300 μM.

The results (**Table 3**) showed that the enzyme possesses the highest activity toward linoleic acid, followed by the α- and then γ-linolenic acids. With regard to acylglycerols, the

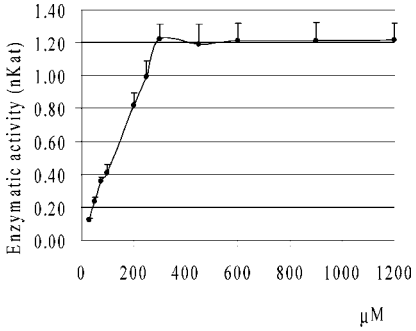

Figure 9. Effect of substrate concentration on *C. intybus* LOX activity.

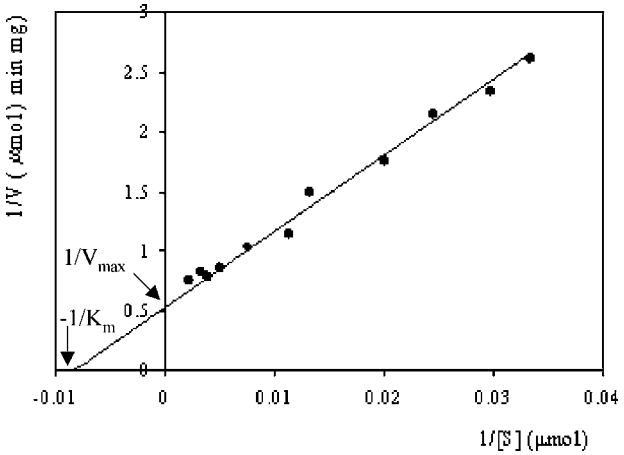

Figure 10. Determination of V_{max} and K_M by Lineweaver–Burk plot.

enzymatic activity was very low when trilinolein was used, whereas no activity was found in the comparison to 1,3-dilinolein. These findings agree with the features found for other vegetable lipoxygenases, which showed the highest activity when the reaction substrates were C18 polyunsaturated fatty acids, linoleic acid being, in particular, the most abundant in plant materials (*2*, *4*).

The effect of the substrate concentration of *C. intybus* LOX was assayed in the linoleic acid concentration range from 30 to 1200 μM using a Tween 20 concentration (0.01 v/v) lower than the critical micelle concentration (*11*, *15*, *41*). **Figure 9** shows that the initial reaction rate increased with linoleic acid concentration in the range of 30−300 μM. At higher linoleic acid concentrations, no increase in enzyme activity was found.

For the determination of *C. intybus* LOX kinetic parameters at optimum pH, ionic strength, and temperature, linoleic acid concentrations were varied from 30 to 300 μM and the data were plotted as a Lineweaver−Burk graph. *C. intybus* LOX K_M and V_{max} values were 1.49×10^{-4} M and 2.049 μM min^{-1} mg^{-1}, respectively (**Figure 10**).

In conclusion, our findings show that a number of soluble compounds found in the Chioggia red chicory possess lipoxygenase activity. Among them, the one with the highest specific activity and the lowest molecular mass was isolated, falling into the molecular mass range generally found for plant lipoxygenases. The enzyme showed high specificity toward linoleic acid; the optimum values of pH, ionic strength, and temperature were 7.58, 30 mM, and 38.5 °C, respectively (**Table 4**). Besides the primary enzymatic activity, hydroperoxide formation, the enzyme showed two secondary enzymatic activities, carotene bleaching activity and the ability to catalyze the hydroperoxide degradation into keto-diene acids.

The features found for this enzyme, that is, the biochemical properties, the kinetic parameters, and the fact that it possesses carotene bleaching activity in aerobic conditions, seem to indicate that the isolated enzyme is a lipoxygenase type III according to the indications given by Siedow for soybean isozymes.

Table 4. Biochemical Properties and Kinetic Parameters of *C. intybus* LOX

pH	7.58
ionic strength	30 mM
temperature	38.5 °C
V_{max}	1.49×10^{-4} M
K_M	2.049 μM min^{-1} mg^{-1}

Investigations are currently under way to isolate and characterize the other soluble LOX isoenzymes occurring in *C. intybus* var. *silvestre*. These results might be useful in establishing the functional role of each individual isoform in the vegetable growth and development and in determining organoleptic properties that are influenced by seasonal temperatures.

ABBREVIATIONS USED

LOX, lipoxygenase; cLOXe, crude enzyme extract; GFC, gel filtration chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IEF, isoelectric focusing; ANOVA, analysis of variance; D (or R) 3 (or 6, 24) h, dialysate (or retentate) obtained after 3 (or 6, 24) h; SD value, standard deviation value.

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