6

Lipoxygenase-Quercetin Interaction: A Kinetic Study Through Biochemical and Spectroscopy Approaches

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1. Introduction

1.1 Lipoxygenase – definition, structure, reaction mechanism and metabolic functions

Lipoxygenases (EC 1.13.11.12, linoleate:oxygen, oxidoreductases, LOXs) which are widely found in plants, fungi, and animals, are a large monomeric protein family with non-heme, non-sulphur, iron cofactor containing dioxygenases that catalyze the oxidation of polyunsaturated fatty acids (PUFA) as substrate with at least one 1Z, 4Z-pentadiene moiety such as linoleic, linolenic and arachidonic acid to yield hydroperoxides (Gardner, 1991).

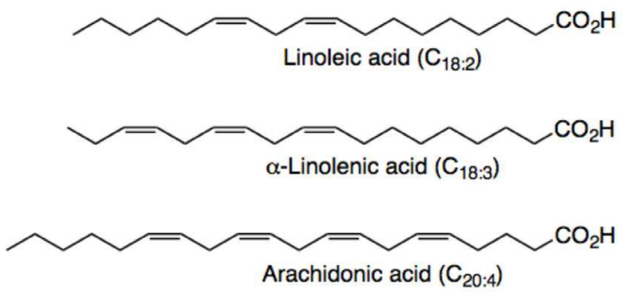

Fig. 1. Lipoxygenase substrates, linoleic, α-linolenic and arachidonic acid.

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The crystal structures of soybean LOX-1 (Minor et al., 1996), LOX-3 (Skrzypczak-Jankun et al., 1997), rabbit 15S-LOX-1 (Gillmor et al., 1997), and coral 8R-LOX (Oldham et al., 2005) have been elucidated thus far and has helped in understanding the properties of LOXs at the molecular level. Several structures of soybean LOX in complex with its product or inhibitor are also known (Skrzypczak-Jankun et al., 2001; Skrzypczak-Jankun[a] et al., 2003; Skrzypczak-Jankun[b] et al., 2003; Borbulevych et al., 2004; Skrzypczak-Jankun et al., 2004). There are significant differences in size, sequence, and substrate preference between the plant and animal LOXs, but the overall folding and geometry of the nonheme iron-binding site are conserved (Kühn et al., 2005; Skrzypczak-Jankun et al., 2006). All LOXs are folded in a two-domain structure that is composed of a smaller β-barrel domain (N-terminal domain) and a larger α-helical catalytic domain (C-terminal domain) (Choi et al., 2008). The nonheme iron essential for activity is positioned deep in a large cavity that accommodates the substrate (Choi et al., 2008). The regio- and stereospecificities of the various LOX isozymes are believed to be determined by the shape and depth of the cavity as well as the binding orientation of the substrate in the cavity (Borngräber et al., 1999; Kühn, 2000; Coffa et al., 2005).

The initial step of LOX reaction is removal of a hydrogen atom from a methylene unit between double bonds in substrate fatty acids (Fig. 2A). The resulting carbon radical is stabilized by electron delocalization through the double bonds. Then, a molecular oxygen is added to the carbon atom at +2 or –2 position from the original radical carbon, forming a peroxy radical as well as a conjugated *trans*, *cis*-diene chromophore. The peroxy radical is then hydrogenated to form a hydroperoxide. The initial hydrogen removal and the following oxygen addition occur in opposite (or antarafacial) sides related to the plane formed by the 1*Z*, 4*Z*-pentadiene unit. In most LOX reactions, particularly those in plants, the resulting hydroperoxy groups are in *S*-configuration, while one mammalian LOX and some marine invertebrate LOXs produce *R*-hydroperoxides. Even in the reactions of such "*R*-LOXs", the antarafacial rule of hydrogen removal and oxygen addition is conserved (Chedea & Jisaka, 2011).

In cases of plant LOXs, including soybean LOXs, the usual substrates are C18-polyunsaturated fatty acids (linoleic and α-linolenic acids), and the products are their 9*S*- or 13*S*-hydroperoxides (Fig. 2B). Most plant LOXs react with either one of the regio-specificity, while some with both. Therefore, based on the regio-specificty, plant LOXs are classified into 9-LOXs, 13-LOXs, or 9/13-LOXs (Chedea & Jisaka, 2011).

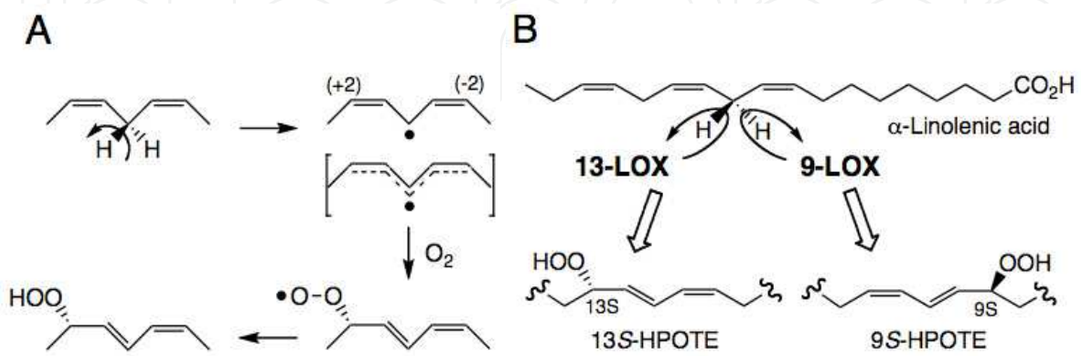


Fig. 2. LOX reaction showing the principal steps of LOX reaction (Panel A), and the actual reactions of plant LOXs and α-linolenic acid (Panel B). HPOTE: hydroperoxyoctadecatrienoic acid (Chedea & Jisaka, 2011).

Theorell et al. (1947) succeeded in crystallizing and characterizing lipoxygenase (LOX) from soybeans and since then among plant LOXs, soybean LOX-1 can be regarded as the mechanistic paradigm for these nonheme iron dioxygenases (Coffa et al., 2005; Minor et al., 1996; Fiorucci et al., 2008).

LOX isoenzymes of soybean seed are 94–97 kDa monomeric proteins with distinct isoelectric points ranging from about 5.7 to 6.4, and can be distinguished by optimum pH, substrate specificity, product formation and stability (Siedow, 1991). LOX-1 is the smallest in size (838 amino acids; 94 kDa), exhibits maximal activity at pH 9.0, and converts linoleic acid preferentially into the 13-hydroperoxide derivative. LOX-2 is characterized by a larger size (865 amino acids; 97 kDa), by a peak of activity at pH 6.8, and forms equal amounts of the 13- and 9-hydroperoxide compounds (Loiseau et al., 2001). LOX-2 oxygenates the esterified unsaturated fatty acid moieties in membranes in contrast to LOX-1 which only uses free fatty acids as substrates (Maccarrone et al., 1994). LOX-3 (857 amino acids; 96.5 kDa) exhibits its maximal activity over a broad pH range centred around pH 7.0 and displays a moderate preference for producing a 9-hydroperoxide product. It is the most active isoenzyme with respect to both carotenoid cooxidation and production of oxodienoic acids (Ramadoss, 1978).

Arachidonic acid metabolism by LOX in platelets was demonstrated in 1974 (Hamberg & Samuelsson, 1974). Rabbit reticulocyte LOX was described for the first time in 1975 (Schewe et al., 1975). There are 6 functional LOXs in humans: 5-LOX; 12/15-LOX (15-LOX-1); platelet-type 12-LOX; 12R-LOX; epidermis-type 15-LOX (15-LOX-2); and epidermis-Alox3. Each of these genes expresses a catalytically active enzyme except epidermis-Alox3, which encodes an enzyme that has hydroperoxidase activity. Thus, the main LOX enzymes with fatty acid oxygenase activity found in humans are 5-LOX, 15-LOX-1, 15-LOX-2, platelet-type 12-LOX and 12R-LOX (Bhattacharya et al., 2009).

In their review concerning the metabolic functions of LOX, Ivanov et al. (2010) present two aspects of LOX biology: (i) 30 years ago, leukotrienes generated by 5-LOX were identified as potent pro-inflammatory mediators (Samuelsson et al., 1980; Jakschik & Lee, 1980). Since then, additional pro-inflammatory products have been discovered (Feltenmark et al., 2008). On the other hand, anti-inflammatory and/or pro-resolving lipids generated by mammalian isoforms have also been identified, including lipoxins (Serhan et al., 1984; Maderna & Gordon, 2009), resolvins (Serhan et al., 2004), protectins (Ariel et al., 2005; Schwab et al., 2007) and maresins (Serhan et al., 2009). Therefore, LOX products play important roles in the development of acute inflammation but they have also been implicated in inflammatory resolution. (ii) Mice deficient in 12R-LOX develop normally during pregnancy but die immediately after birth due to excessive dehydration (Furstenberger et al., 2007; Epp et al., 2007). Although the molecular mechanisms of postpartum mortality are unknown, the enzyme was implicated in the formation of the epidermal water barrier. Genetic polymorphisms of the corresponding human gene have been related to ichthyosis (Eckl et al., 2009), a disease characterized by dry, thickened, scaly or flaky skin (Ivanov et al., 2010).

Available data also suggest that lipoxygenases contribute to *in vivo* metabolism of endobiotics and xenobiotics in mammals (Kulkarni, 2001). Recent reviews describe the role of lipoxygenase in cancer (Bhattacharya et al., 2009; Pidgeon et al., 2007; Moreno, 2009), inflammation (Duroudier et al., 2009; Hersberger, 2010) and vascular biology (Chawengsub

et al., 2009; Mochizuki & Kwon, 2008) and for an extensive presentation of the role of eicosanoids in prevention and management of diseases the reader is referred to the review of Szefel et al. (2011).

1.2 Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) (Fig. 3) is an important dietary flavonoid, present in different vegetables, fruits, seeds, nuts, tea and red wine (Beecher et al., 1999; Formica, 1995; Hollman & Katan, 1999).

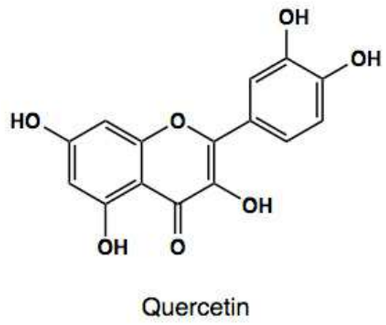


Fig. 3. Chemical structure of quercetin.

Quercetin has been discussed for several decades as a multipotent bioflavonoid with great potential for the prevention and treatment of disease (Bischoff, 2008). Its documented impact on human health includes cardiovascular protection, anticancer, antiviral, anti-inflammatory activities, antiulcer effects and cataract prevention. The study of quercetin as potential chemopreventer is assuming increasing importance considering its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation and metastasis. Quercetin has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells (Lugli et al., 2009).

Among the biological effects of particular relevance, the antihypertensive effects of quercetin in humans and the improvement of endothelial function should also be emphasized (Bischoff, 2008). Together with its antithrombotic and anti-inflammatory effects, the latter mainly mediated through the inhibition of cytokines and nitric oxide, quercetin is a candidate for preventing obesity-related diseases (Bischoff, 2008). Most exciting findings are that quercetin enhances physical power by yet unclear mechanisms. The anti-infectious and immunomodulatory activities of quercetin might be related to this effect (Bischoff, 2008).

Like other flavonoids, quercetin appears to combine both lipoxygenase-inhibitory activities and free radical-scavenging properties in one agent and thus belongs to a family of very effective natural antioxidants (Sadik et al., 2003). Quercetin is a flavonol that can be easily oxidized in an aqueous environment, and in the presence of iron and hydroxyl free radicals (Borbulevych et al., 2004). More specifically, in an aqueous solution, quercetin is known to be oxidized to the relatively stable, neutral protocatechuate intermediate 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2H)-one (Fig. 5). This protocatechuate

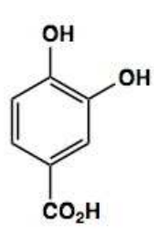
derivative is a common oxidized intermediate mediated by various oxidases, such as lipoxygenase, tyrosinase (Kubo et al., 2004), and peroxidase (Awad et al., 2000), as well as by diphenylpicrylhydrazyl (DPPH) and azobisisobutyronitrile (AIBN) (Krishnamachari et al., 2002) and seems to be a key intermediate to understand quercetin's diverse biological activities (Ha et al., 2010).

1.3 LOX inhibition by quercetin

Because of its unique capability of the direct catalysis the enzymatic lipid peroxidation, 15-LOX-1 belongs to the endogenous prooxidants the action of which may be favored under conditions of oxidative stress (Schewe, 2002). Consequently, the inhibition of 15-LOX-1 may contribute to the universal antioxidant activities of dietary flavonoids (Sadik et al., 2003). Flavonoids appear to combine both lipoxygenase-inhibitory activities and free radical-scavenging properties in one agent and thus constitute a family of very effective natural antioxidants (Sadik et al., 2003). The literature data indicate that quercetin represents one of the most potent inhibitors of different LOXs (Schneider & Bucar[a], 2005; Schneider & Bucar[b], 2005).

The inhibition of rabbit 15-LOX-1 and of soybean LOX-1 by quercetin was studied in detail (Sadik et al., 2003). Quercetin modulates the time course of the lipoxygenase reaction in a complex manner by exerting three distinct effects: (i) prolongation of the kinetic lag period, (ii) instant decrease in the initial rate after the lag phase being overcome, (iii) time-dependent inactivation of the enzyme during reaction, but not in the absence of substrate (Schewe & Sies, 2003). Competitive reversible and irreversible inhibition schemes, as well as inhibition via reduction of the enzyme-bound radical intermediate have been considered to explain the activity of polyphenolic compounds (Fiorucci et al., 2008). Moreover, heterogeneity in the interpretation of the experimental results of the inhibition processes, for example concerning kinetic data, prevents converging toward a general way of inhibition (Fiorucci et al., 2008). It may be supposed that the inactivation is due to combined action of quercetin and intermediates of the catalytic cycle on the active site of the enzyme (Sadik et al., 2003). In previous work, Redrejo-Rodriguez et al. (2004) reported by semiempirical studies that the interaction of quercetin with lipoxygenase is related to the spatial adaptation of the flavonoid to the hydrophobic cavity that constitutes the channel of access of substrate to the catalytic site.

Structural analysis reveals that quercetin entrapped within LOX undergoes degradation and the resulting compound has been identified by X-ray analysis as protocatechuic acid (3,4-dihydroxybenzoic acid) positioned near the iron site (Borbulevych et al., 2004).



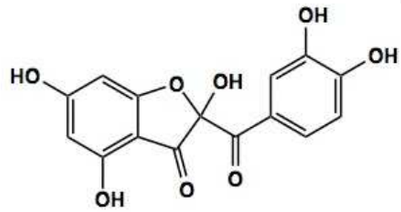
Protocatechuic acid

Fig. 4. Chemical structure of protocatechuic acid, the product of quercetin degradation by soybean LOX-3 as identified by Borbulevych et al. (2004).

The finding that LOX can turn different compounds, like quercetin and epigallocatechin gallate, into simple catechol derivatives (with one aromatic ring only) might be of importance as an additional small piece of a "jigsaw puzzle" in the much bigger picture of drug metabolism. Their interactions with LOX can be more complicated than simply blocking the access to the enzyme's active site (Borbulevych et al., 2004). Ha et al. (2010) have studied the inhibitory activity of protocatechuic acid and of dodecyl protocatechuate on soybean LOX-1 oxidizing linoleic acid. Their results show that the protocatechuate derivative, dodecyl protocatechuate, inhibited the enzymatic peroxidation of linoleic acid as a competitive inhibitor, but its parent compound, protocatechuic acid did not show any activity up to 200 μM. In this way it was shown that the catechol moiety alone is not sufficient to elicit the inhibitory activity and that the hydrophobic dodecyl group is associated with inhibitory activity as it was also reported for dodecyl gallate (Ha & Kubo, 2007; Ha et al., 2010).

Fiorucci et al. (2008) report theoretical investigations concerning three binding modes between quercetin and LOX-3 enzyme. Thus O3, O7, and O4′ oxygen atoms have been considered to bind the iron center (Fiorucci et al., 2008). These specific interactions lead then, through electron transfer from the substrate to the cation, to semiquinone forms, and related tautomeric structures that are compatible with an addition of the triplet spin state dioxygen to the flavonol squeleton (Fiorucci et al., 2008). Among the three considered modes of binding, it appears that quercetin should be linked to the metal center via its 3-OH functional group. The most favorable term to the binding free energy is due to electrostatic interactions (Fiorucci et al., 2008).

In their proposed mechanism of oxidative degradation of quercetin by soybean LOX-1, Ha et al. (2010) indicate that quercetin might first be oxidized to the corresponding o-quinone after the abstraction of 2 e^- and 2 H^+ from the OH groups at C3′ and C4′ (Jungbluth et al., 2000). The enzymatically oxidized o-quinone might be subsequently isomerized to a p-quinone methide type intermediate, followed by the addition of H_2O at C2, yielding the relatively stable intermediate 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2H)-one (Ha et al., 2010). This enzymatically-generated intermediate appears to be relatively stable, and hence, prolongs the inhibitory activity (Ha et al., 2010). The results indicate that the soybean LOX-1 generated intermediate of quercetin is the same as the DPPH (2,2-diphenyl-β-picrylhydrazyl) generated intermediate, 2-(3,4-dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2H)-one (Krishnamachari et al., 2002). In addition, the same oxidized intermediate was also characterized as the relatively stable intermediate generated by mushroom tyrosinase (Ha et al., 2010).



2-(3,4-Dihydroxybenzoyl)-2,4,6-trihydroxybenzofuran-3(2H)-one

Fig. 5. Product of oxidative degradation of quercetin by soybean LOX-1 as proposed by Ha et al. (2010).

The studies on LOX and quercetin contribute to the understanding of biocatalytic properties of this enzyme and its role in the metabolism of this popular (as a medicinal remedy) flavonol and possibly other, similar compounds (Borbulevych et al., 2004).

1.4 O-quinone formation during quercetin oxidation by LOX

Because flavonoids, such as quercetin, and oxidases are present simultaneously in fruits and vegetables, the generation of quinoid derivatives in biological systems is plausible (Pinto & Macias, 2005). This process is of great relevance from a biological point of view, because the conversion of supposed beneficial antioxidants such as flavonoids to electrophilic prooxidants may constitute a possible toxicological risk (Boersma et al., 2000).

On the other hand, it is to be noted that, in addition to dioxygenase activity, lipoxygenase, possesses a peroxidase activity toward a wide range of compounds (Gardner, 1991). Dioxygenase activity produces the insertion of oxygen into a polyunsaturated fatty acid containing a 1,4-*cis*,*cis*-pentadiene moiety, producing the corresponding lipid hydroperoxide. In the process, an intermediate peroxyl radical is generated. This compound, or the peroxide, supports the cooxidase activity of lipoxygenase toward a suitable electron donor, which is transformed into a radical (Pinto & Macias, 2005). The hydroperoxidase activity of LOX also can be observed in the presence of hydrogen peroxide instead of lipid hydroperoxide, being related to xenobiotic oxidation processes (Hover & Kulkarni, 2000; Santano[a] et al., 2002; Santano[b] et al., 2002). In addition, it is known that a variety of phenolic compounds and flavonoids with antioxidant properties are inhibitors of lipoxygenase (Prasad et al., 2004).

On the basis of the results obtained, Pinto & Macias (2005) concluded that in the presence of hydrogen peroxide or hydroperoxylinoleic acid, lipoxygenase produces a quinoid product as a result of the enzymatic oxidation (in the presence of hydrogen peroxide) or cooxidation (in the presence of linoleic acid) of quercetin (Pinto & Macias, 2005).

During lipoxygenase catalysis enzyme-bound prooxidant intermediates such as fatty acid peroxyl radical ($ROO^{\bullet}$) are formed (Schewe, 2002). It is tempting to speculate, therefore, that the flavonoids are co-oxidized in this system to a semi-quinone or quinone (with flavonoids containing a catechol B ring) or a phenoxy radical (with noncatechol flavonoids) which in turn may covalently bind to sulfhydryl or amino groups of the lipoxygenase, thus rendering its inhibition irreversible (Sadik et al., 2003). In the case of quercetin and other flavonols, the intermediate formation of corresponding quinone methides (Awad et al., 2001) may be involved (Sadik et al., 2003).

Quercetin is considered an excellent free radical scavenging antioxidant owing to the high number of hydroxyl groups and conjugated π orbitals by which quercetin can donate electrons or hydrogen, and scavenge H_2O_2 and superoxide anion ($\bullet O^{2-}$) (Heijnen et al., 2001). The reaction of quercetin with $\bullet O^{2-}$ leads to the generation of the semiquinone radical and H_2O_2 (Metodiewa et al., 1999). Quercetin also reacts with H_2O_2 in the presence of peroxidases, and thus it decreases H_2O_2 levels and protects cells against H_2O_2 damage; nevertheless, during the same process potentially harmful reactive oxidation products are also formed. The first oxidation product of quercetin is a semiquinone radical (Metodiewa et al., 1999). This radical is unstable and rapidly undergoes a second oxidation reaction that produces another quinone (quercetin-quinone, QQ) (Metodiewa et al., 1999). Since QQ can

react with proteins, lipids and DNA, it is responsible for protein and DNA damage as well as lipid peroxidation.

The oxidative decomposition of quercetin by hydroperoxidase activity of lipoxygenase has been reported, suggesting that in the presence of the lipoxygenase/H_2O_2 system quercetin is oxidized to a quinoid product. It is remarkable that this behavior is not shown by naringenin or resveratrol, other bioactive antioxidant phenolics, probably due to the different redox potentials of these compounds (Pinto & Macias, 2005).

UV-Vis spectroscopy is a widespread and commonly used technique that has been successfully used for the determination of catalytic mechanism, including enzyme-substrate/inhibitor interaction profiles. In this report, we used UV-Vis spectroscopy to help elucidate possible interacting/inhibitory effect of quercetin with soybean LOX-1, oxidizing or not the linoleic acid. The UV-Vis spectral analysis of the mixture quercetin and LOX-1 and the kinetic parameters (K_m and V_{max}) of LOX-1-catalyzed oxidation of linoleic acid in the absence and presence of different quercetin concentrations revealed that:

i. quercetin (λ_{max}= 370 nm) was oxidized to a new compound having λ_{max}= 321 nm by LOX-1 in absence of substrate.
ii. a mixed inhibition occurred for quercetin concentrations in the range of 10~50 μM.
iii. at 100 μM, the highest quercetin concentration tested, the K_{mapp} decreased by half while reaction rate increased indicative of a cooxidation of quercetin in addition to the LOX classical reaction illustrating the switch in quercetin's role from inhibitor towards substrate.
iv. quercetin concentration significantly affected its partitioning level as a substrate or an inhibitor of LOX.
v. the ratio substrate:inhibitor might be a factor determining the type of inhibition observed in the case of lipoxygenase and quercetin interaction.

2. Biochemical analysis of LOX interaction with quercetin

2.1 UV-Vis spectroscopy

When a sample of an unknown compound is exposed to light, certain functional groups within the molecule absorb light of different wavelengths. In UV/Visible Spectroscopy, the term chromophore is used to indicate a functional group that absorbs electromagnetic radiation, usually in the UV or visible region. The type of functional groups that absorb ultraviolet light can be conjugated species, such as alkenes, aromatics, etc., making UV/Visible spectroscopy useful for distinguishing conjugated dienes from conjugated trienes, and so forth (Perkampus, 1992).

The reference beam in the spectrometer travels from the light source to the detector without interacting with the sample. The sample beam interacts with the sample exposing it to ultraviolet light of continuously changing wavelength. When the emitted wavelength corresponds to the energy level which promotes an electron to a higher molecular orbital, energy is absorbed. The detector records the ratio between reference and sample beam intensities (I_0/I). At the wavelength where the sample absorbs a large amount of light, the detector receives a very weak sample beam. Once intensity data has been collected by the spectrometer, it is sent to the computer as a ratio of reference beam and sample beam

intensities. The computer determines at what wavelength the sample absorbed a large amount of ultraviolet light by scanning for the largest gap between the two beams (Perkampus, 1992).

When a large gap between intensities is found, where the sample beam intensity is significantly weaker than the reference beam, the computer plots this wavelength as having the highest ultraviolet light absorbance when it prepares the ultraviolet absorbance spectrum. Once the spectrometer has collected data from sample exposure to the UV beam, the data is transmitted to an attached computer which processes the intensity/wavelength data to produce an absorbance spectrum (Perkampus, 1992).

Various techniques have been devised for the determination of lipoxidase activity, including colorimetric, polarographic and spectrophotometric methods (Holman, 1955). Firstly, the spectrophotometric method was developed after Holman and Burr (1945) and Bergström (1946). They had independently observed an increase in ultraviolet light absorption, at 234 nm, when lipoxidase acted upon essential fatty acids. The increase in UV-peak absorption was then related to the amount of peroxide formation which, was found to be proportional to time and to enzyme concentration (Tappel, 1962; Theorell et al., 1944). The polyunsaturated fatty acid is solubilized by the addition of a detergent, and with this soluble substrate the activities of purified and crude lipoxidase are demonstrated over a wide range of pH (Tappel, 1962).

In the '50s the research done for the elucidation of lipoxygenase activity was strongly linked to the UV-Vis spectrometry and until today the UV-Vis spectrometry is an essential tool in probing the lipoxygenase activity. Thus in 1952 in an article published in the Journal of Biological Chemistry, Tappel et al. (1952), using a Beckman DU quartz spectrophotometer that was equipped with a temperature-controlled cell compartment, showed that with sodium linoleate as a substrate, under suitable conditions the reaction velocity was linear with respect to the enzyme concentration and the reaction did not show an induction period. With methyl linoleate the reaction velocity was not directly proportional to enzyme concentration unless a Tween preparation was added.

Antioxidants inhibited linoleate oxidation as a result of a direct effect on lipoxidase and of a preferential oxidation of the antioxidant. Rapid oxidation of nordihydroguaiaretic acid under suitable conditions was obtained in the absence of net linoleate oxidation, the linoleate having a function analogous to that of a coenzyme (Tappel et al., 1952).

The inactivation of soybean lipoxygenase during oxygenation of fatty acid substrates was first described by Theorell et al. (1944). It was shown that velocity of the lipoxygenase-catalyzed reaction decreases as a linear function of substrate utilization with all substrates tested.

2.2 UV-Vis spectra of LOX reaction with quercetin

We demonstrated by UV-Vis spectroscopy that pH values may influence the molecular interactions between soybean LOX-1 and quercetin, and especially the alkaline pH favors the ionic display of quercetin in order to interact with LOX better (Chedea et al., 2006). After 60 min of incubation of soybean LOX and quercetin (50 μM) the UV-Vis spectra showed the formation of a new product (λ_{max} = 321 nm) indicating the formation of an intermolecular complex between LOX and quercetin (Fig. 6) (Chedea et al., 2006).

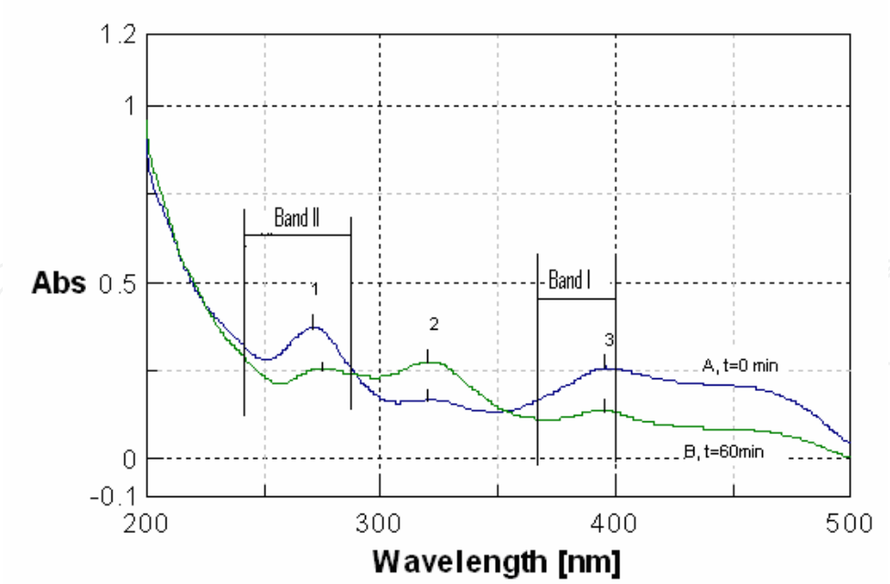


Fig. 6. The absorption spectra of the mixture soybean LOX-1 and quercetin (50 μM) at the initial moment (min 0), blue line, and after 60 min of incubation, green line. For measurements 160 μl standard LOX-1 (2300 enzymatic units/ml) and quercetin to the final concentration of 50 μM were added to 790 μl 0.2 M phosphate buffer pH 9. The spectra were registered on a Jasco-V 500 spectrophotometer and the blank contained 950 μl buffer and 50 μl ethanol (Chedea et al., 2006).

Fig. 6 shows the spectra of the mixture of LOX and quercetin. Three peaks (1, 2, 3) registered at t=0 and t=60 min of incubation were identified. Peak 1 corresponds to Band II, peak 3 to Band I, both bands characteristic for flavonoids, while peak 2 indicated the formation of a new compound as the result of reaction between lipoxygenase and quercetin.

In the spectral range of 240-450 nm, flavone and their hydroxy substituted derivatives show two main absorption bands commonly referred to as Band I (300-400 nm) and Band II (240-280 nm) (Mabry et al., 1970). Band I is supposed to be associated with the light absorption of the cinnamoyl system (B+C ring), and Band II with the absorption of the benzoyl moiety formed by the A+C ring (Fig. 7) (Zsila et al., 2003).

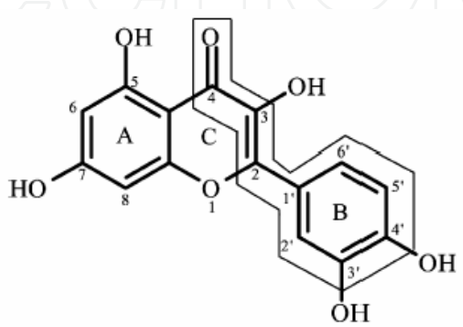


Fig. 7. Chemical structure of quercetin. Frame highlights the cinnamoyl part of the molecule (Zsila et al., 2003).

The results of Chedea et al. (2006) are confirmed by the study of Ha et al. (2010). They observed the decrease in the absorbance of the band centered at 272 and 386 nm with the concomitant increase in the absorbance at 330 nm, and the presence of two isosbestic points at 286 and 357 nm, respectively, which is also in agreement with the report of Takahama (Takahama, 1985), and the hypsochromic shift likely indicates that quercetin was oxidized (Ha et al., 2010). The absorbance at 385 nm mainly results from the $n \rightarrow \pi^*$ transition, and hence, the shift may be caused by structural changes including the ketone moiety, besides the change of the catechol on the ring B to the corresponding o-quinone (Ha et al., 2010). In the study of Pinto and Macias, when quercetin is incubated in the presence of lipoxygenase and linoleic acid, a decrease of the band centered at 375 nm is produced, together with a slight increase of absorbance in the region of 330-340 nm (Pinto & Macias, 2005).

Lipoxygenase inhibition, as a result of intermolecular interaction between the enzyme and quercetin, is dependent on the quercetin's concentration. As the concentration of inhibitor increases the formation of a new compound as a result of this reaction increases as well, showing that a greater concentration of quercetin determines a more intense interaction between lipoxygenase and inhibitor (Vicaş et al., 2006). Quercetin exhibited a dose-dependent inhibitory effect, and the lipoxygenase-catalyzed oxidation of linoleic acid to 13-HPOD was inhibited with an IC_{50} value of 4.8 ± 4 μM (Ha et al., 2010).

Pinto at al. (2011) have shown also the existence of the interaction between lipoxygenase and quercetin. They also investigated the formation of an intermolecular complex between quercetin and lipoxygenase (Pinto et al., 2011). The acting forces between lipoxygenase and quercetin include mainly hydrogen bond and van der Waals, electrostatic and hydrophobic forces (Pinto et al., 2011).

2.3 Enzyme kinetics

Enzyme kinetics is the investigation of how enzymes bind substrates and turn them into products. The rate data used in kinetic analyses are obtained from enzyme assays. In 1913 Leonor Michaelis and Maud Menten proposed a quantitative theory of enzyme kinetics, which is referred to as Michaelis-Menten kinetics. Their work was further developed by G. E. Briggs and J. B. S. Haldane, who derived kinetic equations that are still widely used today.

The major contribution of Michaelis and Menten was to think of enzyme reactions in two stages. In the first, the substrate binds reversibly to the enzyme, forming the enzyme-substrate complex. This is sometimes called the Michaelis-Menten complex in their honor. The enzyme then catalyzes the chemical step in the reaction and releases the product (Rogers & Gibon, 2009).

To find the maximum speed of an enzymatic reaction, the substrate concentration is increased until a constant rate of product formation is seen. Saturation happens because, as substrate concentration increases, more and more of the free enzyme is converted into the substrate-bound ES form. At the maximum velocity (V_{max}) of the enzyme, all enzyme active sites are saturated with substrate, and the amount of ES complex is the same as the total amount of enzyme.

However, V_{max} is only one kinetic constant of enzymes. The amount of substrate needed to achieve a given rate of reaction is also important. This is given by the Michaelis-Menten

constant (K_m), which is the substrate concentration required for an enzyme to reach one-half its maximum velocity. For finding the substrate concentration required for an enzyme to reach one-half its maximum velocity, the reaction speed is measured at different substrate concentrations. Each enzyme has a characteristic K_m for a given substrate, and this can show how tight the binding of the substrate is to the enzyme (Rogers & Gibon, 2009).

The effect of substrate concentration ([S]) on activity is usually expressed using a Michaelis-Menten plot, such as the one shown below, and enzymes which generate such a plot are said to obey Michaelis-Menten kinetics. Michaelis-Menten plots show three distinct regions which correspond to reaction order. At low [S], the reaction accelerates as more substrate is added, reflecting first-order kinetics. At high [S], the concentration of enzyme becomes limiting, and additional substrate cannot accelerate the reaction. This situation is known as zero-order kinetics. Finally, there is a transition period between first order and zero order where kinetics are mixed (http://wiz2.pharm.wayne.edu/biochem/enz.html).

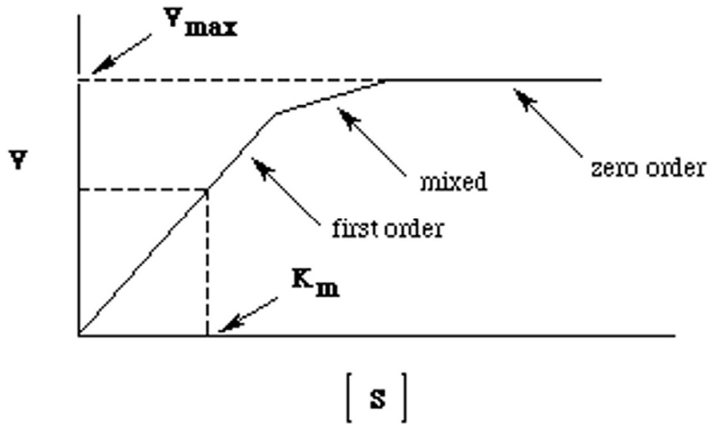


Fig. 8. A Michaelis-Menten plot.

If one draws a line across from the level (zero order) region of the plot to the Y-axis, this data point is V_{max}, the maximum rate of reaction for a given concentration of enzyme. The second kinetic constant is also derived by drawing a line from the Y-axis at 1/2 V_{max} to the curve, and then down to the X-axis. Each substrate will generate a unique K_m and V_{max} for a given enzymatic process.

A standard equation used to express the kinetic constants under the Michaelis-Menten hypothesis is aptly called the Michaelis-Menten equation, and is shown below. Later, two other investigators rearranged this equation to generate a second useful equation, the Lineweaver-Burk equation, also shown below.

Two things should be noticed about the Lineweaver-Burk equation: first, it is in the form y = mx + b, and as such, a plot of this equation will generate a straight line for enzymes obeying simple Michaelis Menten kinetics. In addition, the x and y values for the plot are both inverted, and as such, the plot is often referred to as the double reciprocal plot. The Lineweaver-Burk plot has two advantages over the Michaelis-Menten plot, in that it gives a more accurate estimate of V_{max}, and it gives more accurate information about inhibition as

The Michaelis-Menton Equation

$$V = \frac{Vmax\,[S]}{Km + [S]}$$

The Lineweaver-Burke Equation

$$\frac{1}{V} = \frac{K_m}{V_{max}}\,\frac{1}{[S]} + \frac{1}{V_{max}}$$

well. A typical Lineweaver-Burk plot appears below. Note that V_{max} is derived from the y-intercept, and K_m can be derived either from the slope, or from extrapolating the line to the negative X-axis (http://wiz2.pharm.wayne.edu/biochem/enz.html).

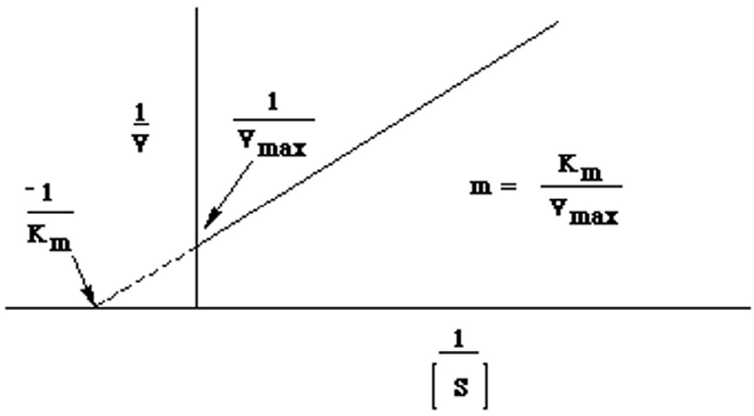


Fig. 9. A typical Lineweaver-Burk plot.

2.3.1 Enzyme inhibition

There are two main classes of enzyme inhibitors, reversible and irreversible, that are differentiated by the magnitude of their affinity for enzyme. Reversible enzyme inhibitors bind and dissociate with their enzyme in an equilibrium process. Irreversible inhibitors bind tightly to an enzyme to form an essentially permanent complex. Reversible inhibitors can be classified as competitive, mixed, or noncompetitive inhibitors. If the detailed mechanism of inhibition is known, then the classification can be made by identifying where on the enzyme the inhibitor binds, or the order with which it binds, relative to substrate. Alternatively, a determination of simple kinetic parameters can generally be used to classify the inhibitor (http://www.wiley.com/college/pratt/0471393878/student/animations/enzyme_inhibition/index.html)

2.3.1.1 Competitive inhibitors

Competitive inhibitors compete with substrate for an enzyme's active site, lowering the enzyme's likelihood of binding substrate and slowing the observed reaction velocity. Kinetic studies can be used to determine the type and potency of inhibition for an unknown

inhibitor. Typical steady-state kinetic experiments can be performed where reaction velocity is measured in the presence of varying concentrations of substrate. If inhibitor is then added, and the data shows an increase in K_m, yet the V_{max} is unaffected, this is the signature of a competitive inhibitor (http://www.wiley.com/college/pratt/0471393878/student/animations/enzyme_inhibition/index.html)

2.3.1.2 Mixed inhibition

A mixed inhibitor binds to a site on the enzyme and interferes with both apparent substrate affinity and catalytic turnover, thus affecting the observed K_m for the enzyme-catalyzed reaction. Mixed inhibitors do not bind directly in the active site, and therefore do not block substrate binding, but instead bind at sites that can be proximal or distal from the active site. Mixed inhibitors can therefore bind to free enzyme prior to substrate, distorting the active site to a nonoptimal conformation for catalysis. The inhibitor-distorted active site has trouble converting the substrate to product before it dissociates, resulting in a lowered apparent substrate binding affinity. Steady-state experiments performed in the presence of a mixed inhibitor demonstrate an increase or decrease in K_M, and a decrease in V_{max} (http://www.wiley.com/college/pratt/0471393878/student/animations/enzyme_inhibition/index.html)

2.3.1.3 Noncompetitive inhibition

Noncompetitive inhibition is a special case of mixed inhibition where the affinity of inhibitor for E and ES is the same. Steady-state experiments performed in the presence of a noncompetitive inhibitor demonstrate a decrease in V_{max}, yet K_m is unaffected (http://www.wiley.com/college/pratt/0471393878/student/animations/enzyme_inhibition/index.html)

2.4 The influence of pure quercetin on sodium linoleate oxidation by pure soybean LOX-1

2.4.1 The influence of quercetin on the LOX-1 oxidation of sodium linoleate for different experimental protocols

It is of great interest to check if the oxidative decomposition of quercetin by lipoxygenase is produced in the presence of linoleic acid. It is known that the hydroperoxidase activity of lipoxygenase produces the cooxidation of suitable electron donors in the presence of the hydroperoxides of linoleic or arachidonic acid, the natural substrates for this enzyme (Pinto & Macias, 2005). When linoleic acid is used as the substrate the primary LOX dioxygenation product obtained is (9Z,11Z,13S)-hydroperoxyoctadeca-9,11-dienoic acid (13-HPOD) (Grechkin, 1998).

Dioxygenase activity of lipoxygenase was measured by recording the increase in absorbance at 234 nm (formation of 13-HPOD), the incubation mixture containing 50 μl quercetin in ethanol to a final concentration of 100 μM, 8.4 μl sodium linoleate at different concentrations (2.2 mM, 4.39 mM, 6.6 mM and 20 mM) and 160 μl lipoxygenase (2300 units/ml) in 782 μl 0.2 M borate buffer pH 9. The final substrate concentrations were 18.5 μM, 36.9 μM, 55.4 μM respectively 168 μM. The reaction components were mixed following three protocols as Fig. 10 indicates. The differences between these protocols are given by the order in which the enzyme, the substrate and the inhibitor were mixed and the incubation time.

As already presented in section 2.2, lipoxygenase in the absence of linoleic acid interacts with quercetin, thus Protocol 1 was designed to establish how the inhibition of substrate oxidation occurs when LOX-1 is initially incubated with the inhibitor. On the hand, Protocol 2 follows the classical way of LOX assay where the enzyme reacts with the substrate in the presence of quercetin. In the typical LOX reaction, the oxidation of the iron atom occurs with consumption of one molecule of fatty acid hydroperoxide (Zheng & Brash, 2010), therefore, the objective of the protocol was to explore the possibility of quercetin reacting with the LOX reaction product, the 13-hydroperoxide. Finally, the objective of Protocol 3 was to check for the existence of competition between the substrate and inhibitor in a reaction environment where both linoleic acid and quercetin-also oxidized by lipoxygenase-exist.

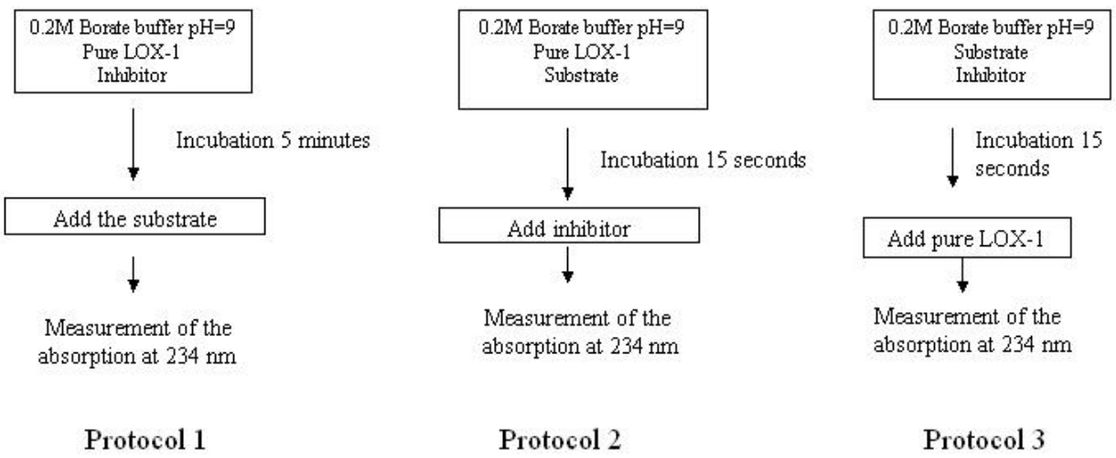


Fig. 10. Schematic representation of experimental protocols (1,2,3).

Fig. 11. presents comparatively the inhibitory effect of quercetin 100 μM on the oxidation of sodium linoleate by LOX-1 using all 3 experimental protocols. The kinetic curves of the reaction in the presence of quercetin are compared with the one of lipoxygenase without inhibitor (red curve).

It can be seen from Fig. 11 that the strongest inhibition is reached in the case of protocol 2 (pink line) when the inhibitor was added after the incubation for 15 seconds of sodium linoleate 55.4 μM with the enzyme. For protocol 1 the typical Michaelis-Menten kinetic plot shape was maintained like for the LOX reaction with substrate without inhibitor (Fig. 11 blue curve vs. red plot). A different shape has the plot in the case of protocols 2 (pink line) and 3 (green line). At low substrate concentrations for both protocols, 2 and 3 (Fig. 11 curves green and pink), the kinetic plots start with a burst phase-a very fast increase of the reaction velocity. It follows a decrease which is fast in the case of protocol 3 (green curve) and slower in the case of protocol 2 (pink curve). For protocol 2 the kinetic curve continues with a slight increase (starting with 55.4 μM concentration of substrate), which in our situation didn't reach the plateau phase. In the case of protocol 3 a new increase of the reaction rate -not so high compared with the first burst- follows at substrate concentration between 36.9 μM and 55.4 μM, continuing with a slight decrease at concentrations of substrate higher than 55.4 μM.

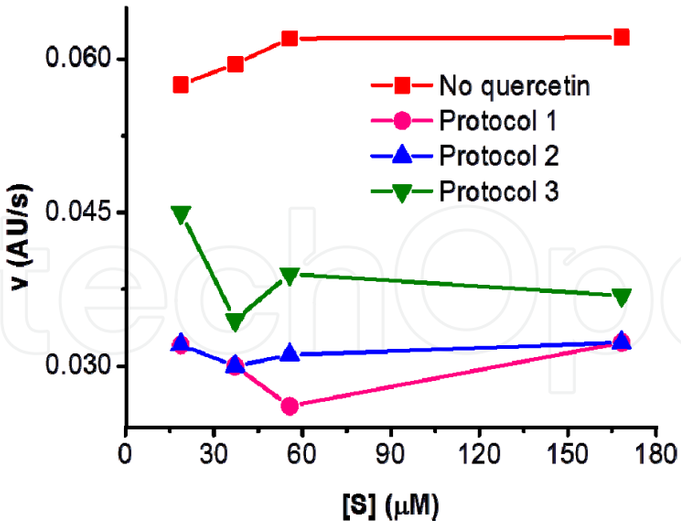


Fig. 11. The inhibitory effect of quercetin on the lipoxygenase reaction according to protocol 3- dark green line; to protocol 2- pink line; to protocol 1- blue line. The red curve represents the kinetic without quercetin. The reaction components, 782 μl 0.2 M borate buffer, (pH = 9.0), 160 μl of LOX solution (2300 U enzyme/ml) 8.4 μl of sodium linoleate, of different concentrations (2.2 mM, 4.39 mM, 6.6 mM and 20 mM) having the final substrate concentration in the reaction mixture of 18.5 μM, 36.9 μM, 55.4 μM and 168 μM) and 50 μl quercetin in ethanol to the final concentration of 100 μM were added and mixed as Fig. 10. presents. The absorption at 234 nm (A_{234}) against the blank was recorded.

When both the substrate and the inhibitor were mixed and left for few seconds and then LOX was added the kinetic curve (Fig. 11 protocol 3), the first phase of fast increase shows that the hydroperoxides are formed as oxidation product of the linoleic acid even though the quercetin is present in the reaction mixture for very low substrate concentrations. LOX is partially inhibited by the quercetin at 36.9 μM substrate concentration but then, at higher concentrations of substrate the enzyme becomes active again, but not as much as in the case of very low substrate concentration. Rapid inhibition followed by time dependent inactivation of soybean LOX-1 was also observed by Sadik et al. when quercetin was added to the reaction set-up after the substrate (Sadik et al., 2003).

When LOX was incubated with the substrate for 15 seconds and then the quercetin was added, the kinetic plot shows that LOX oxidizes the substrate having the highest product yield for very low substrate concentrations, that quercetin partially inhibits the reaction at linoleate concentrations between 30 μM and 55 μM.

Careful examination of the three protocols leads to speculation the quercetin-based inhibition of the soybean LOX-1 oxidation of linoleic acid does not follow the typical competitive inhibition model under the experimental conditions used (comparatively, protocol 3 gave the lowest inhibition than the other protocols tested). In Protocol I, it was observed that the new compound, a LOX-1-catalyzed by-product of quercetin, effectively inhibited the linoleic acid's oxidation. In the case where quercetin is added the last, the LOX reaction inhibition pattern suggested that at certain substrate concentrations (between 45 μM and 75 μM) quercetin would react with the hydroperoxide responsible for the initiation

of the LOX cycle, and thus implying the possibility of the quercetin undergoing non-enzymatic oxidation leading to the reaction inhibition.

2.4.2 Determination of kinetic parameters K_m and V_{max} of soybean LOX-1 standard towards linoleic acid as substrate

The kinetic parameters for LOX-1 oxidation of sodium linoleate were calculated as control values and compared with LOX-1 oxidation of sodium linoleate in the presence of different concentrations of quercetin. For the measurements of pure LOX activity, to 832 µl 0.2 M borate buffer, (pH = 9.0), 160 µl of LOX solution (2300 U enzyme/ml) and 8.4 µl of sodium linoleate of different concentrations (2.2 mM, 4.39 mM, 6.6 mM and 20 mM), having the final substrate concentration in the reaction mixture of 18.5 µM, 36.9 µM, 55.4 µM and 168 µM, were added. The absorption at 234 nm (A_{234}) against the blank was recorded. The blank contained a mixture of 840 µl 0.2 M borate buffer, (pH = 9.0) and 160 µl of LOX solution (2300 U enzyme/ml). In order to obtain the Lineweaver-Burk plot, the v, 1/[S] and 1/v were calculated for different substrate concentrations (from 18.5- 168 µM).

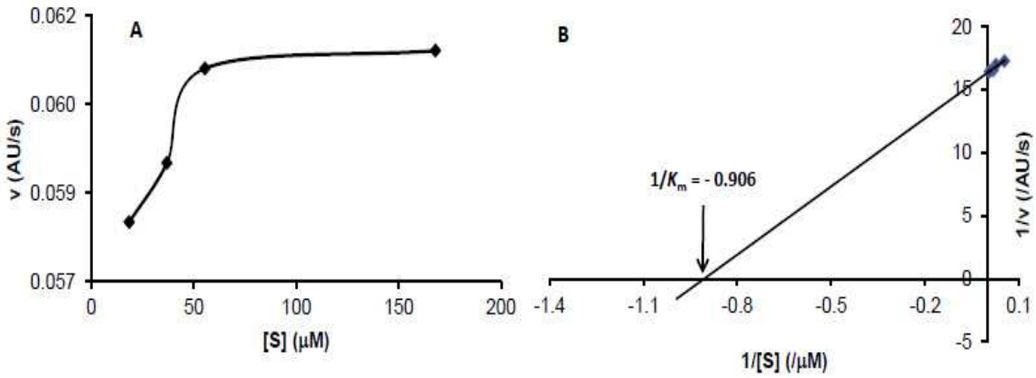


Fig. 12. Michaelis-Menten plot (A) and Lineweaver-Burk plot (B) for different sodium linoleate concentrations oxidized by standard LOX-1. For the measurements of pure LOX activity, to 832 µl 0.2 M borate buffer, (pH = 9.0), 160 µl of LOX solution (2300 U enzyme/ml) and 8.4 µl of sodium linoleate of different concentrations (2.2 mM, 4.39 mM, 6.6 mM and 20 mM), having the final substrate concentration in the reaction mixture of 18.5 µM, 36.9 µM, 55.4 µM and 168 µM, were added. The absorption at 234 nm (A_{234}) against the blank was recorded. The blank contained a mixture of 840 µl 0.2 M borate buffer, (pH = 9.0) and 160 µl of LOX solution (2300 U enzyme/ml) (Chedea et al., 2008). Excel program was used to draw the graphs.

The kinetic parameters calculated for LOX-1 oxidizing sodium linoleate show that the enzyme has a great affinity towards substrate (K_m = 1.1 µM) and that its oxidation is fast as well (V_{max}= 2.7 μMs^{-1}).

2.4.3 Kinetic parameters for LOX-1 oxidizing sodium linoleate in presence of different quercetin concentrations

The reaction velocities in the case of this first protocol for the three quercetin concentrations were calculated as the ratio of absorption and correspondent time registered at 234 nm. For

the absorption measurements of pure LOX activity to 782 µl 0.2 M borate buffer, (pH = 9.0), 160 µl of LOX solution (2300 U enzyme/ml) 50 µl quercetin (10 µM, 50 µM and 100 µM final concentrations) and 8.4 µl of sodium linoleate of different concentrations (2.2 mM, 4.39 mM, 6.6 mM and 20 mM), having the final substrate concentration in the reaction mixture of 18.5 µM, 36.9 µM, 55.4 µM and 168 µM, were added. The absorption at 234 nm (A_{234}) against the blank was recorded. The blank contained a mixture of 840 µl 0.2 M borate buffer, (pH = 9.0) and 160 µl of LOX solution (2300 U enzyme/ml).

The inhibitory effect of quercetin at different concentrations (100, 50 and 10 µM) is presented in Fig. 13.

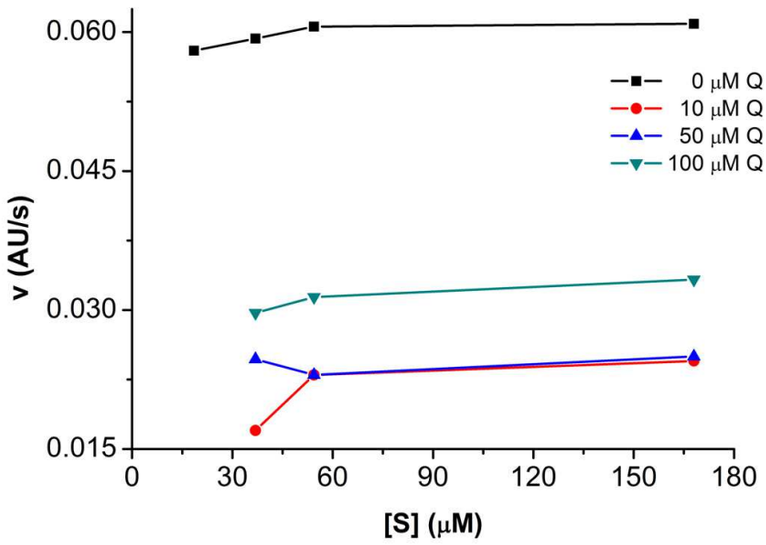


Fig. 13. Lipoxygenase inhibition by quercetin at different concentrations (10 µM, 50 µM and 100 µM). Protocol 1 (Fig. 10) was followed for measurements as indicated in the legend of Fig. 11.

Plotting the reaction velocity in function of substrate concentration it can be seen that the classical Michaelis-Menten shape of the curve is not registered in the case of LOX reaction inhibition by quercetin at 50 µM. For this reason, it was taken into calculation a larger range of substrate and quercetin concentration and for those the reaction velocity was calculated.

The kinetic curves representing the reaction velocity of the sodium linoleate oxidation by LOX-1, in presence of different concentrations of quercetin (Fig. 13) show that quercetin has an inhibitory effect on lipoxygenase for all the concentrations tested. To obtain these results, the reaction components were mixed as protocol 1 indicates, the enzyme being incubated with quercetin for 5 minutes and then the substrate is added. From Fig. 14 it can be seen that none of the kinetic plots follows the classical Michaelis-Menten shape. To get more information about the quercetin inhibitory action on soybean LOX-1, the kinetic parameters were calculated.

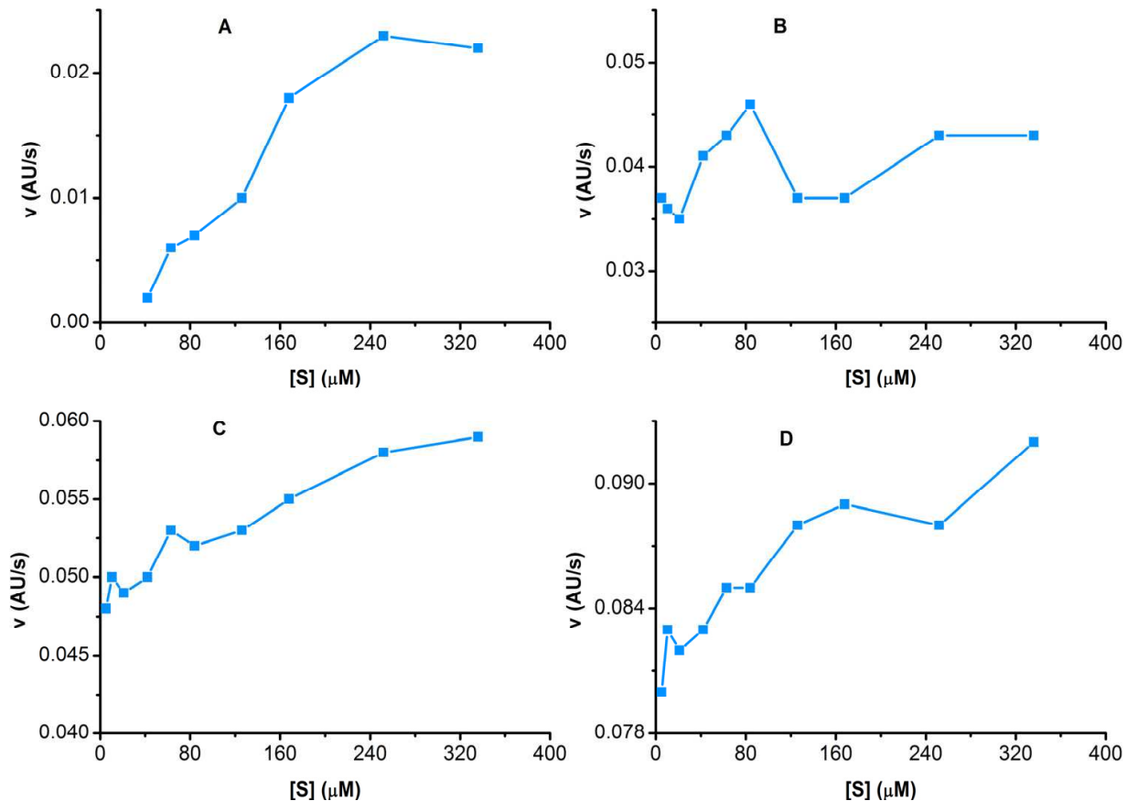


Fig. 14. Kinetic plots of the sodium linoleate (336 μM, 252 μM, 168 μM, 126 μM, 84 μM, 63 μM, 42 μM, 21 μM, 10.5 μM and 5.25 μM), oxidation by LOX-1, in presence of different concentrations of quercetin 10 μM (A), 25 μM (B), 50 μM (C) and 100 μM (D).

Fig. 14 indicates different behaviours of LOX-1 quercetin inhibition determined by the substrate's and inhibitor's concentrations. For quercetin at 100 μM (Fig. 14D), 50 μM (Fig. 14C), at low concentrations of substrate (up to 21 μM a fast increase of the reaction velocity is recorded, followed by a slight decrease and increase again (concentration of substrate between 21 μM and 42 μM). This "oscillatory" behaviour continues at higher substrate concentrations in the case of quercetin at 100 μM and 50 μM with a tendency of increasing the reaction's velocity at concentrations of substrate higher than 336 μM for 100 μM quercetin and of reaching the plateau phase for 50 μM quercetin.

A fast decrease followed by an increase until the maximum velocity, characterises the kinetic plot of LOX-1 oxidizing the sodium linoleate at low substrate concentrations up to 84 μM (Fig. 14B) in the presence of quercetin 25 μM. At this concentration of substrate (84 μM) the reaction velocity reaches its maximum. It follows a significant decrease of the reaction velocity at substrate concentrations between 84 μM and 134.4 μM, the reaction rate increasing again and having the tendency to follow the classical Michael-Menten kinetic shape till at 252 μM, substrate's concentration. At this point a slight decrease of the reaction rate is registered, showing that for quercetin 25 μM the inhibition is given by the high substrate concentration as well, when the sodium linoleate has a concentration higher than 252 μM (Fig. 14B). The same behaviour is registered in the case of quercetin 10 μM at linoleate concentrations higher than 252 μM (Fig. 14A). For quercetin 10 μM the kinetic plot

follows the tendency of the classical Michaelis-Menten shape, with a slight decrease in reaction velocity at concentrations of substrate between 63 μM and 126 μM.

The observation that diminution of the degree of inhibition occurred by the decreasing of substrate concentration may imply that the substrate oxidation also plays a role in the process of LOX-1 inhibition by quercetin. This involvement may be one of the initial steps in the LOX inhibition, where quercetin is non-enzymatically oxidized by the reaction's product, the hydroperoxide, resulting in the formation of the quercetin o-quinone. Once the "activation" of quercetin through its oxidation is triggered, it can be assumed that the enzymatic inhibition would proceed via a quercetin oxidation product.

K_m, K_{mapp} and V_{max} calculated from the Lineweaver-Burk plots, for LOX-1 oxidizing sodium linoleate in the absence and presence of quercetin at 10 μM, 25 μM, 50 μM and 100 μM are presented in Table 1.

Quercetin conc. μM	K_{mapp} (μM)	V_{max} ($\mu Ms^{-1}/s$)	Type of inhibition
100	0.55	3.8	-
50	0.83	2.4	mixed
25	0.78	1.8	mixed
10	261.78	0.5	mixed
0	1.103	2.7	

Table 1. K_m, K_{mapp} and V_{max} calculated from the Lineweaver-Burk plots, for LOX-1 oxidizing sodium linoleate in the absence and presence of quercetin at 10 μM, 25 μM, 50 μM and 100 μM.

The determined K_{mapp} and V_{max} show a mixed inhibition for quercetin concentrations in the range of 10-50 μM. For 100 μM the K_{mapp} is half decreased and the reaction rate increases, reaching its highest value.

In a previous study LOX activity was measured spectrophotometrically at 234 nm using 15 mU of enzyme in the presence of 100 μM linoleic acid in 50 mM potassium phosphate buffer, pH 7.5. For each scan 2 μl of quercetin solution was added to 3 mL lipoxygenase solution to give a final concentration in the range 0.8 to 4.0 μM and an inhibitory effect concentration dependent was detected (Pinto et al., 2011). This effect shows the characteristics of a competitive mechanism as it was deduced from Lineweaver-Burk plot (Pinto et al., 2011). On the basis of the competitive inhibition detected, the interaction should be located near or at the catalytic site (Pinto et al., 2011). The results obtained from the evaluation of three dimensional florescence spectra suggest a conformational modification of the protein in the region of the coupling with quercetin (Pinto et al., 2011).

The degree of inhibition was paradoxically diminished with decreasing substrate concentration which reveals an unusual mode of the inhibitory effect (Sadik et al., 2003). In this case, a competitive type of inhibition appears to be excluded (Sadik et al., 2003). The rabbit reticulocyte 15-LOX-1 samples were pre-incubated for 2 min at 20° with 10 μM quercetin and the reactions were started by addition of 0.265 mM potassium linoleate and the formation of conjugated dienoic fatty acids was recorded spectrophotometrically at 234 nm (Sadik et al., 2003). Ha et al. report that quercetin inhibited soybean LOX-1 in a non-

classical manner. The progress curves of O_2 consumption showed that quercetin inhibited soybean LOX-1 by a slow-binding inhibition mechanism (Ha et al., 2010).

3. Conclusions

The aim of this study was to show how a widespread analysis tool like UV-Vis spectroscopy shapes the enzyme inhibition research having as an example the lipoxygenase interaction with quercetin. Almost all therapeutic drugs are enzyme inhibitors, from old medicine box standards such as aspirin and penicillin to the newest compounds used to treat HIV infection. Understandably, enzyme kinetics plays an outstanding role in this effort to produce effective therapeutics, for kinetic studies can quantify the degree that inhibitors inactivate or slow down the targeted enzyme's catalytic rate and describe its potential efficacy as a drug.

Since its characterisation in 1947 lipoxygenase is strongly related to the UV-Vis spectrometry as a valuable tool for its activity assay. Due to its implications in food chemistry and medicine LOX inhibition attracted up to date, the interest of researchers in the field. A highly functionalized flavonoid, quercetin proves to be a potent inhibitor of lipoxygenase acting both as a substrate and a source of inhibition, quercetin seems to play an antinomic role (Fiorucci et al., 2008). The partitioning level between quercetin as a substrate or as an inhibitor is dependent on its concentration. Different reversible inhibition schemes (competitive, noncompetitive, or mixed) as well as inhibition via reduction of the enzyme-bound radical intermediate have been considered to explain the activity of quercetin as LOX inhibitor. Moreover, heterogeneity in the interpretation of the experimental results of the inhibition processes, for example concerning kinetic data, prevents converging toward a general way of inhibition (Fiorucci et al., 2008). The ratio substrate:inhibitor might be a factor determining the type of inhibition observed in the case of lipoxygenase and quercetin interaction.

In our present study the UV-Vis spectra show the oxidation of quercetin and the formation of a new compound. The absorption maximum of this new formed molecule is centered around λ_{max}= 321 nm, different from quercetin (λ_{max}= 370 nm), suggesting loss of π-electron delocalisation, i.e. interruption of the quercetin B and C ring π-bond extended conjugated system (Bors et al., 1990; Abou Samra et al., 2011). The determined K_{Mapp} and v_{max} show a mixed inhibition for quercetin concentrations in the range of 10-50 μM. For the highest quercetin concentration tested, 100 μM, the K_{mapp} decreased by half but the reaction rate increases which might indicate a cooxidation of quercetin besides the LOX classical reaction, proving the switch in quercetin role from inhibitor towards substrate. Recent literature data show that quercetin itself inhibits the LOX reaction and also its oxidation products are inhibitors of LOX oxidation. For instance, quercetin may first act as a lipoxygenase inhibitor by reducing the ferric form of the enzyme to an inactive ferrous form, and then, the oxidized metabolite becomes a more potent inhibitor (Ha et al., 2010).

In our study, the Michaelis-Menten (M-M), Lineweaver-Burk kinetics and UV spectral analysis have detected both the mixed inhibition and cooxidation of quercetin during soybean LOX-1-mediated metabolism of polyunsaturated fatty acid, linoleic acid. Though not yet convincingly proven, the mechanisms underlying the distinct kinetic behaviors are, at least empirically, believed to be due to the existence of divergent interactions between the

quercetin molecules and/or its intermediates and the active site of the enzymes. Furthermore, the atypical kinetics might be interpreted using the model of two or possibly more binding regions within the enzyme active site(s) for the quercetin and intermediates. The finding that LOX can turn different compounds, like quercetin and epigallocatechin gallate, into simple catechol derivatives (with one aromatic ring only) might be of importance as an additional small piece of a "jigsaw puzzle" in the much bigger picture of drug metabolism (Borbulevych et al., 2004). However, the interactions of these flavanols with LOX can be more complicated than simply blocking the access to the enzyme's active site as observed in this study. Therefore, it warrants future endeavours to thoroughly understand thus reliably predict interaction mechanism between the LOX proteins and therapeutic agents at the molecular level. This will be important in fully understanding the exact role of lipoxygenase inhibition by quercetin in therapy targeting and possibly identifying new bioactive molecules which would be used as drugs.

4. Acknowledgment

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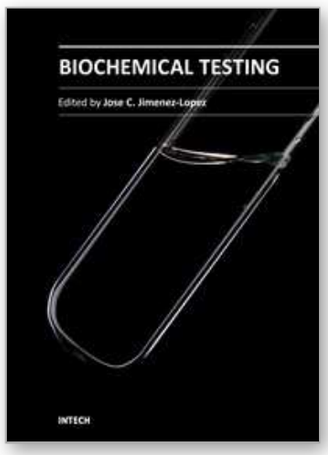

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Biochemical testing necessitates the determination of different parameters, and the identification of the main biological chemical compounds, by using molecular and biochemical tools. The purpose of this book is to introduce a variety of methods and tools to isolate and identify unknown bacteria through biochemical and molecular differences, based on characteristic gene sequences. Furthermore, molecular tools involving DNA sequencing, and biochemical tools based in enzymatic reactions and proteins reactivity, will serve to identify genetically modified organisms in agriculture, as well as for food preservation and healthcare, and improvement through natural products utilization, vaccination and prophylactic treatments, and drugs testing in medical trials.

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