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# Kinetic of inhibition of lipoxygenase in presence of natural amino acid serine

R.N. Raykova[1], E. Carvalho[2], L.S. Manovski[1], D.L. Danalev*[1], D.A. Marinkova[1], T.I. Pajpanova[3], S.A.Yaneva[4], L.K. Yotova[1]

[1]University of Chemical Technology and Metallurgy, Biotechnology Department, Sofia 1756, Bulgaria

[2]University of Cergy-Pontoise, France

[3]Institute of Molecular Biology "Roumen Tsanev", Bulgarian Academy of Sciences, Sofia 1113, Bulgaria

[4]*University of Chemical Technology and Metallurgy, Department of Fundamentals of Chemical Technology Sofia 1756, Bulgaria*

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Lipoxygenase (LOX) is an enzyme, found in many plants and animals, which catalyses the oxygenation of polyunsaturated fatty acids (PUFA) to form fatty acid hydroperoxides. The latter are present in a wide range of biological organs and tissues, particularly abundant in grain legume seeds (beans and peas) and potato tubers. Lipoxygenase from different sources, catalyses oxygenation at different points along the carbon chain, which is referred as regio - specificity. Such specificity has significant implications for the metabolism of the formed hydroperoxides into a number of important secondary metabolites. According to the literature, it is known that primary alcohols and amines inhibit lipoxygenase activity. Herein, we report our studies on inhibitory effect of natural amino acid serine on lipoxygenase, isolated from avocado. The affinity of LOX is higher with L-Ser, compared to D-Ser. It means that natural amino acid is more compatible for the enzyme, but at the same time Ki values reveal that D-Ser has stronger inhibitory effect against LOX from avocado.

L-Serine shows competitive type of inhibition against LOX and for D-Serine the inhibition type of enzyme catalyzed reaction is mixed.

**Key words**: Lipoxygenase, inhibitors, serine, amino acid

## INTRODUCTION

Lipoxygenases catalyze the oxidation of unsaturated fatty acids and have a wide range of biomedical applications. In mammals, LOX aid in the production of leukotrienes and lipoxins, which regulates responses in inflammation and immunity [1, 2]. Thus, LOX inhibitors have been used as drug agents for treatment of inflammatory diseases such as asthma, atherosclerosis, and psoriasis [3]. In addition, LOX inhibitors are promising cancer chemotherapeutics [4-7]. Numerous kinetic studies of LOXs have been carried out using soybean LOX-1 [8] and human LOX [9] with linoleic acid (LA) as a substrate [10]. They all reveal the same mechanism of action (Figure 1), where the pro-*S* hydrogen atom from carbon atom C11 of LA is transferred to the Fe(III)−OH cofactor, forming a radical intermediate substrate and Fe(II)−$OH_2$ [8, 11]. Authors also described that a subsequent reaction with molecular oxygen could be further realized and it eventually leads to hydroperoxyoctadecadienoic acid and Fe (III)−OH [8].

LOX pathway has become important therapeutic target for prevention of different inflammatory diseases and cancer treatments. The Food and Drug Administration (FDA) approved drugs for diseases, caused by LOX, but some of them have been reported to exhibit various side effects [12]. Hence, it is essential to implement specific inhibitor which will not interfere with the other normal physiological functions (Fig. 2).

In this global context finding new inhibitors of LOX could be a promising alternative as chemotherapeutics for prevention and treatment of different diseases, where LOX plays a key role.

LOX can be inhibited by several natural products, including flavonoids [14, 15], thiourea and the derivatives [16-18], etc. Rioux N. et al. conclude in their work that alcohols and primary amines are good LOX inhibitor [7]. Taking into account this information, we decided to study the effect of amino acid serine for its ability to inhibit LOX.

## EXPERIMENTAL

### *Chemicals*

Linolenic acid was purchased from Sigma Aldrich. L- and D-Serine were obtained from Riedel-de haen. Isolation and purification of avocado LOX and its characteristics (activity, pH

* To whom all correspondence should be sent:
E-mail: dancho.danalev@gamail.com

and temperature optimum) were previously described in [19]. 0.1M acetate buffer with pH 6.5 was used for all kinetic studies.

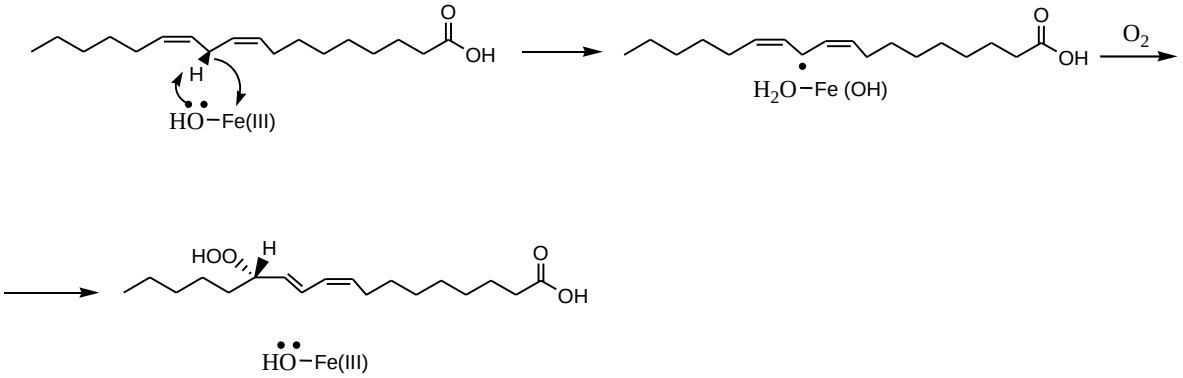



**Fig. 1**. Proposed mechanism of soybean lipoxygenase [8]. The net hydrogen atom transfer from the linoleic acid substrate to the Fe (III) − OH cofactor is on focus.

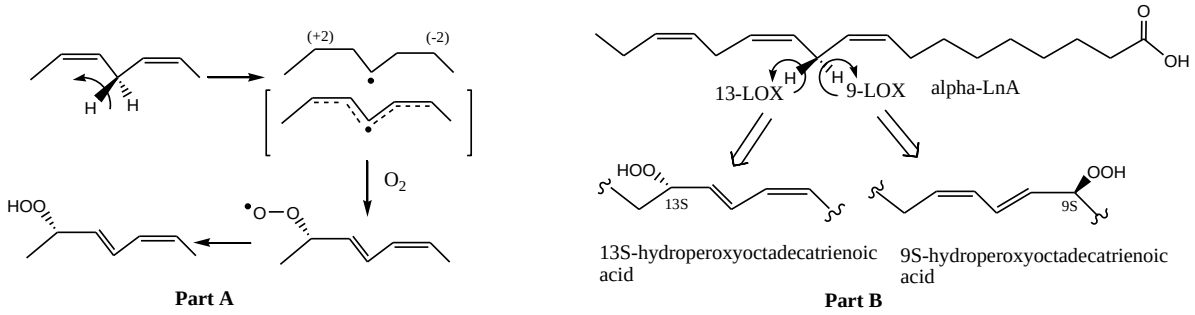



**Fig.2.** LOX reaction showing the principal steps of LOX reaction (Part A), and the actual reactions of plant LOXs and α-linolenic acid (Part B) [13].

## KINETIC STUDIES

### *Substrate preparation*

0.1 ml linolenic acid (LnA) was dissolved in 60 ml 95% EtOH. The obtained solution was diluted to 100 ml with distilled water and stirred for 1 hour. Before activity measurement, the needed quantity was diluted 5 times with 0.2 M acetate buffer, pH 6.5 [20].

### *Enzyme solution preparation*

Enzyme solution with concentration 1 mg/ml in 0.1 M acetate buffer, pH 6.5, was prepared. Before measurement the protein content was diluted 5 times up to final concentration 20 μg/ml [20-22].

### *Determination of avocado LOX kinetics*

Kinetics of enzyme catalyzed reactions in presence of avocado LOX and LnA were measured spectrophotometrically on Perkin Elmer Lambda 2 spectrophotometer at 234 nm.

Michaelis constant, $K_m$ and $V_{max}$ were calculated using Lineweaver-Burk plot (L-B plot) [23, 24]. Different concentrations of LnA for the enzyme catalyzed reactions were investigated and applied.

### *Kinetics of inhibition of avocado LOX*

Control sample of 2.4 ml solution of inhibitor and 0.1 ml substrate solution was prepared. Concerning the sample, 2.1 ml solution of inhibitor and 0.1 ml substrate solution were incubated. At zero time 0.3 ml enzyme with concentration 20 μg/ml was added and the measurement of ΔA/min was obtained by UV-spectrophotometer at 234 nm for 10 min, taking into account the linear range of reactions [25].

## RESULTS AND DISCUSSION

Serine is a natural amino acid including both primary amino and primary hydroxyl functions (Figure 3).

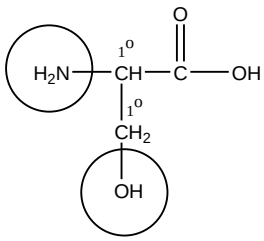



**Fig. 3.** Serine structure

Structurally serine includes all necessary moieties, according to Rioux N. et al., to be a

potent LOX inhibitor [7]. Taking into account this information, we investigated kinetics of inhibition of avocado LOX in presence of L- and D-Serine.

For our purpose substrate solutions with several concentrations (Table 1) were prepared, starting from a stock solution described in experimental section.

The obtained curve for reaction kinetics, in absence of inhibitor for avocado LOX and presence of LnA as a substrate, is presented on Figure 4.

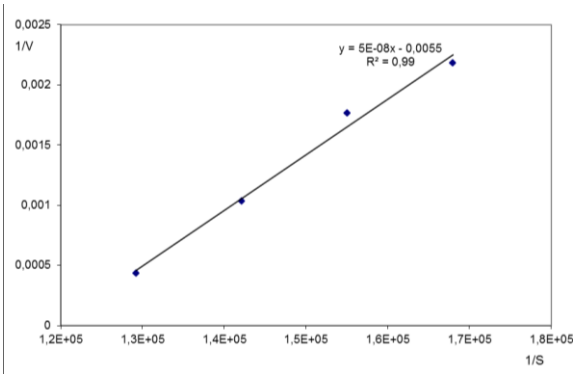



**Fig. 4.** L-B plot for avocado LOX kinetics with linolenic acid

Calculated kinetic parameters, according to the presented L-B plot are: Km 9,09.$10^{-6}$ M and Vmax 181,82 M.$sec^{-1}$.

Further, our work continued with determination of kinetic parameters in presence of L- and D-Serine as inhibitors. For this purpose, inhibitor solutions with several concentrations were prepared (Table 2).

In order to determine type of inhibition in presence of L-Ser the following concentrations of substrate and inhibitor were applied: initial substrate concentrations:2,69.$10^{-3}$M; 2,94.$10^{-3}$M; 3,23.$10^{-3}$M; 3,59.$10^{-3}$M; initial L-Ser concentrations: 0,6.$10^{-3}$M; 1,0.$10^{-3}$M; 3,0.$10^{-3}$M.

The obtained data for kinetic reactions with both inhibitors are presented on Figure 5 and 6, respectively.

The obtained results show a competitive type of inhibition, which can be also noticed from the calculated $K_i$ and $K_m$ parameters – in cases of competitive inhibition $K_i$ shows lower values than $K_m$, overcoming the effect of increased substrate concentration.

In order to calculate $K_i$, $K_m$ and $V_{max}$ the following concentrations were used: initial substrate concentrations: 2,69.$10^{-3}$M; 2,94.$10^{-3}$M; 3,59.$10^{-3}$M; initial L-Ser concentrations: 0,3.$10^{-3}$M; 0,6.$10^{-3}$M; 1,0.$10^{-3}$M; 3,0.$10^{-3}$M.

By applying Dixon plot, we determined the following values for kinetic parameters in presence of L-Serine: $K_i$ 1,1394.$10^{-5}$ M, $K_m$ 2,26.$10^{-5}$ M and $V_{max}$ 0,2005 M.$sec^{-1}$.

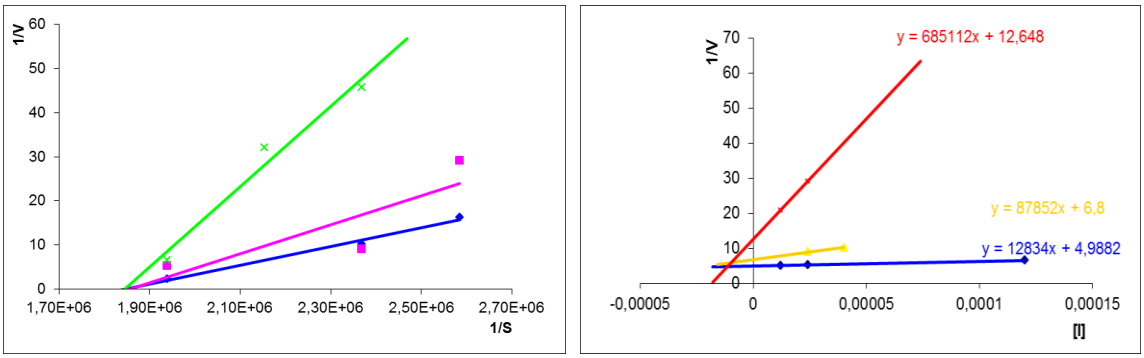
The probable role of L-Ser as a drug agent, taking into account the expressed competitive inhibition, might be to increase the intracellular concentration of LnA and in that way to accumulate the substrate and inhibit its utilization if needed.

**Table 1.** Substrate concentrations used for enzyme kinetic measurements.

| Substrate | Stock solution concentration, $10^{-3}$M | Solution concentration in cuvette (kinetics in absence of inhibitor), $10^{-6}$ M | Solution concentration in cuvette (kinetics in presence of inhibitor), $10^{-5}$ M |
|---|---|---|---|
| LnA | 3,59 | 8,60 | 0,052 |
| | 3,23 | 7,74 | 0,046 |
| | 2,94 | 7,04 | 0,042 |
| | 2,69 | 6,45 | 0,039 |

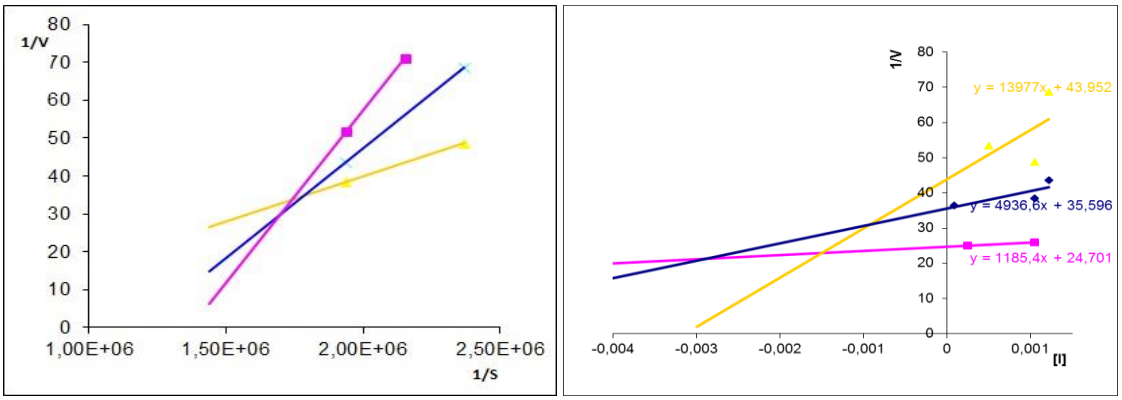
**Table 2.** Concentrations of LOX inhibitors, used for determination of type of inhibition and calculation of kinetic parameters of the enzyme catalyzed reactions.

| Inhibitor | Stock solution concentration, $10^{-3}$M | Solution concentration in cuvette, $10^{-5}$ M |
|---|---|---|
| L-Ser | 0,3 | 12,0 |
| | 0,6 | 24,0 |
| | 1,0 | 40,0 |
| | 3,0 | 120,0 |
| D-Ser | 0,1 | 8,0 |
| | 0,3 | 25,2 |
| | 0,6 | 50,4 |
| | 1,45 | 121,8 |




**A** **B**

**Fig. 5.** Kinetics of inhibition of LOX in presence of L-Ser: **A.** L-B double reciprocal plot for determination of type of inhibition; **B.** Dixon plot for determination of kinetic parameters.




**A** **B**

**Fig. 6.** Kinetics of inhibition of LOX in presence of D-Ser: **A.** L-B double reciprocal plot for determination of type of inhibition; **B.** Dixon plot for determination of kinetic parameters.

**Table 3.** Values of kinetic parameters in presence of inhibitors in case of avocado LOX reaction

| Inhibitor | Ki [M] | Km [M] | Vmax [M.sec$^{-1}$] |
|---|---|---|---|
| L-Serine | 1,14.10$^{-5}$ | 2,26.10$^{-5}$ | 0.2005 |
| D-Serine | 1,91.10$^{-6}$ | 6,23.10$^{-5}$ | 0,1239 |
| Absence of inhibitor | - | 9,09.10$^{-6}$ | 181,82 |

In order to determine the type of inhibition of D-Ser against avocado LOX the precise concentrations were used: initial substrate concentrations 2,69.10$^{-3}$M; 2,94.10$^{-3}$M; 3,23.10$^{-3}$M; 3,59.10$^{-3}$M; initial D-Ser concentrations 0,3.10$^{-3}$M; 0,6.10$^{-3}$M; 1,45.10$^{-3}$M. The obtained results reveal a mixed type of inhibition, where typical is increasing of $K_m$ value (substrate binding) and decreasing of $V_{max}$ value (hamper catalysis).

Further, applying Dixon plot representation, we prepared: initial substrate concentrations 2,94.10$^{-3}$M; 3,23.10$^{-3}$M; 3,59.10$^{-3}$M; initial D-Ser concentrations 0,1.10$^{-3}$M; 0,3.10$^{-3}$M; 0,6.10$^{-3}$M; 1,45.10$^{-3}$M and determined the following values of kinetic parameters, representing the qualitative effect of the inhibitor against LOX: $K_i$ 1,91.10$^{-6}$ M, $K_m$ 6,23.10$^{-5}$ M and $V_{max}$ 0,1239 M.sec$^{-1}$.

All data for calculated parameters is summarized in Table 3.

Most of the pharmaceutical inhibitors are competitive and a few are mixed ones, lowering Vmax and increasing Km. For this reason, D-Ser is hopeful alternate inhibitor for LOX reactions and their place in cell processes.

## CONCLUSIONS

Km values in case of L- and D-Ser inhibition process show that the affinity of LOX from avocado is higher in presence of L-Ser, compared to D-Ser. It means that natural amino acid is highly recognized and interfered to the active site of the enzyme, but Ki values reveal that D-Ser shows significantly stronger inhibitory effect against LOX.

The type of inhibition in cases of L- and D-Ser differs. The effect of L-Serine occurs in competitive inhibition, whilst D-Ser inhibiting mechanism is mixed.

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# КИНЕТИКА НА ИНХИБИРАНЕ НА ЛИПОКСИГЕНАЗА В ПРИСЪСТВИЕ НА ПРИРОДНАТА АМИНОКИСЕЛИНА СЕРИН

Р.Н.Райкова[1], Е.Карвальо[2], Л.С.Мановски[1], Д.Л.Даналев[1], Д.А.Маринкова[1], Т.И.Пайпанова[3], С.А.Янева[4], Л.К. Йотова[1]

*[1]Химикотехнологичен и металургичен университет, катедра "Биотехнология", София 1756, България*
*[2]Университет на Cergy-Pontoise, Франция*
*[3]Институт по молекулярна биология „Румен Цанев", Българска академия на науките, София 1113, България*
*[4]Химикотехнологичен и металургичен университет, катедра "Основи на химичните технологии", София 1756, България*

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(Резюме)

Липоксигеназата (ЛГ) е ензим, който се среща в много растения и животни и катализира процеса на окисление на полиненаситените мастни киселини (PUFA) до образуване на техни хидропероксиди. Тя присъства в големи количества в биологични органи и тъкани и по-специално е в изобилие в зърната на бобовите растения (боб и грах) и картофите. ЛГ от различни източници катализира процеса на окисление в различни части от въглеродната верига, т.е. притежава региоселективност. Това има огромно значение за метаболизирането на образуваните хифропероксиди в някои важни вторични метаболити. Според литературни данни първични алкохоли и амини инхибират липокзигеназната активност. В настоящата работа ние докладваме изследвания за инхибиторният ефект на природната аминокиселина серин (Ser) върху ЛГ, изолирана от авокадо. Нашите изследвания показаха, че афинитета на ЛГ е по-висок към L-Ser в сравнение с D-Ser. Това означава че природната форма на аминокиселината е по-съвместима с ензима, но в същото време Ki стойностите показват, че D-Ser притежава по-мощен инхибиторен ефект. L-Ser показва конкурентен тип на инхибиране, докато при D-Ser той е от смесен тип.