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Development of novel peptide inhibitor of Lipoxygenase based on biochemical and BIAcore evidences

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ABSTRACT

Lipoxygenase (LOX) are enzymes implicated in a broad range of inflammatory diseases, cancer, asthma and atherosclerosis. These diverse biological properties lead to the interesting target for the inhibition of this metabolic pathway of LOX. The drugs available in the market against LOX reported to have various side effects. To develop potent and selective therapeutic agents against LOX, it is essential to have the knowledge of its active site. Due to the lack of structural data of human LOX, researchers are using soybean LOX (sLOX) because of their availability and similarities in the active site structure. Based on the crystal structure of sLOX-3 and its complex with known inhibitors, we have designed a tripeptide, FWY which strongly inhibits sLOX-3 activity. The inhibition by peptide has been tested with purified sLOX-3 and with LOX present in blood serum of breast cancer patients in the presence of substrate linoleic acid and arachidonic acid respectively. The dissociation constant (K_D) of the peptide with sLOX-3 as determined by Surface Plasmon Resonance (SPR) was 3.59×10^{-9} M. The kinetic constant (K_i) and IC_{50}, as determined biochemical methods were 7.41×10^{-8} M and 0.15×10^{-6} M respectively.

1. Introduction

The Lipoxygenase pathway has become important therapeutic target for the prevention of different inflammatory diseases and in cancer chemoprevention. So far, the Food and Drug Administration (FDA) approved drugs for diseases caused by LOX reported to have various side effects [1]. Hence, it is essential to implement specific inhibitor which will not interfere with the other normal physiological functions.

The oxidative metabolism in arachidonate pathway, fatty acids produce prostaglandins, hydroxyl fatty acids and leukotrienes, which are collectively referred as *eicosanoids*. These fatty acids are the substrate for three distinctively different enzymatic pathways, Cyclooxygenase (COX), LOX and Epoxygenase. LOX are class of non-heme iron enzyme found in both plant and animals. In plant, LOX's are involved in germination and senescence [2,3]. The sLOX has been the most extensively studied enzyme of all the Lipoxygenase. Soybean contains at least three distinct isozymes, sLOX-1, sLOX-2 and sLOX-3. The sLOX-1 and sLOX-3 enzymes are very similar in their behavior and mostly are considered together as a single type. These isozymes exhibit diversity in their enzymatic behavior, their regio-specificities and pH optima. The crystal structure of sLOX 1 and 3 are known so far. In animals it is one of the key enzymes in the metabolism of arachidonic acid to hydroperoxyeicasotetranoic acid (HETE) and subsequent conversion to leukotriene. Leukotrienes are potent mediator of variety of inflammatory diseases and cancers [4–7]. Mammalian LOXs are classified as 5-, 8-, 12-, and 15-Lipoxygenases depending on the position of oxygen insertion during arachidonic acid oxygenation.

The active site of animal LOX is similar to that of plant LOX with regards to its sequence homology and structure as seen by comparing rabbit LOX with soybean LOX [8–13]. The highest level of sequence identity between LOX from plants and mammals lies in the area of the catalytic domain containing the non-heme iron atom. Use of soybean LOX as a model for designing inhibitors will prove highly beneficial and a tool in structural characterization, mechanism elucidation and possibly the discovery of novel class of peptide inhibitors [14].

The known LOX inhibitors and other NSAIDs act by blocking the conversion of arachidonic acid to eicosanoids. Some of these eicosanoids are involved in the normal physiological function and also for inflammatory diseases. Thus designing specific inhibitor of LOX will help to control the inflammatory pathways.

It has been observed from the literature that most of the known inhibitors contain aromatic residues with hydroxyl group. The substrate linoleic acid, arachidonic acid and the product HETE and hydroperoxyoctadecadienoic acid (HPOD) interacts with aromatic group of Trp^{500} (π–π interactions) and with positively charged amino acid residues like Arg^{707} (charge–charge interactions) [15].

Abbreviations: LOX, Lipoxygenase; sLOX, soybean LOX; PLA_2, Phospholipase A_2; K_D, dissociation constant; SPR, Surface Plasmon Resonance; K_i, kinetic constant; FDA, Food and Drug Administration; COX, Cyclooxygenase; HETE, hydroperoxyeicasotetranoic acid; HPOD, hydroperoxyoctadecadienoic acid; RU, Resonance Unit; DMF, dimethylformamide; HBTU, 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-3 (diethylaminopropyl) carbodiimide

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In last few years all the companies are in a race to identify the small molecule drug that would make it in the market first. Peptide leads to new class of chronic pain drug [16]. Peptides play important role in the regulation of many physiological processes as peptides acts as neurotransmitters or growth factors (hormones) in endocrine or paracrine manner. Peptides bind to specific receptors and catalytic site and regulate the function [17,18] and can be used to generate specific inhibitors against target proteins [19]. In the present study we report the biochemical and SPR evidences of a tripeptide FWY as a potent inhibitor of LOX.

2. Results

2.1. Purification and characterization of LOX

The soybean LOX enzyme was purified after extraction from soybean seeds by two chromatographic steps: First anion exchange, in which five peaks were obtained by a linear gradient formed by the 10 mM potassium phosphate buffer, pH 6.8 and 200 mM potassium phosphate buffer, pH 6.8. Peaks 1–5 (Fig. 1a) were different proteins eluted at different concentration gradients. Out of these, peak 4 showed sLOX activity when analyzed. Peak 1, 2, 3 and 5 were other proteins of different molecular weights which have shown no sLOX activity. In second step of cation exchange, two peaks were obtained and the peak eluted at the buffer gradient 25 mM MES buffer containing 175 mM NaCl, pH 5.8 shows sLOX activity. The purification and SDS profiles for all steps are shown in Fig. 1a, b, c and d. The activity profile obtained for the purified enzyme shows that LOX was highly active (Fig. 2a). The maximal velocity was obtained within first 30s and was constant for several seconds. Specific activity for the enzyme was calculated as 86.54 U/mg protein. The purified protein band was excised from gel and identified through Mass Spectrometry. The trypsanized fragments were analyzed through a mascot search program from matrix science shows candidate proteins as sLOX-3 and a score of 263 based on probability analysis. The score of 263 for an MS/MS match in Mascot search depends upon the absolute probability that the selected match between reported data and the database sequence is a random event.

2.2. Screening of peptides

The activity assay of sLOX-3 in presence of 15 different synthesized peptides was performed using UV spectrophotometer. Table 1 shows the decrease in the activity of the enzyme (percentage inhibition) when the assay was performed in the presence of the peptides (enzyme activity was considered 100% when assay was performed without any inhibitor). In presence of peptide FWY activity was decreased to more than 85%. The inhibition studies for peptide FWY was also performed with serum from breast cancer patient and showed more than 75% inhibition of animal LOX activity with using substrate arachidonic acid. The assay was performed at 234 nm which gives the absorption maxima for the products 5-HETE, 12-HETE, 15-HETE and 269 nm for LTB_4.

2.3. Determination of K_D by surface plasmon resonance

The Fig. 3a and b shows sensorgram for binding of peptide FWY and linoleic acid at varying concentrations with immobilized sLOX on sensor chip. The change in Resonance Unit (RU) with varying concentrations of peptide indicated the change in bound mass on sensor surface with time and the dissociation constant was found to be 3.59×10^{-9} M and 6.56×10^{-5} M for peptide FWY and Linoleic acid respectively. The binding of FWY was stronger than that of substrate

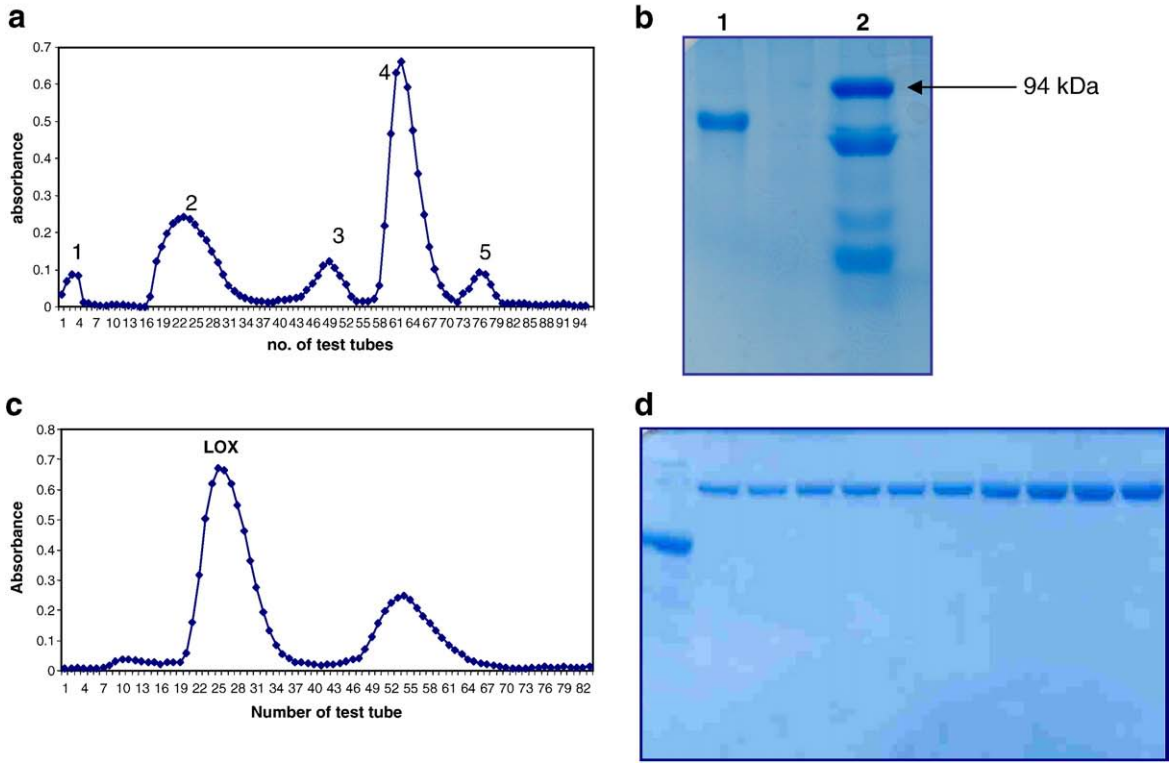

Fig. 1. (a) Elution profile of Soybean Lipoxygenase after DEAE-Sephadex A-50 anion exchange column chromatography. Peak 4 corresponds to the fraction containing sLOX-3 activity. (b) SDS-PAGE profile of Soybean Lipoxygenase after DEAE-Sephadex A-50 anion exchange column chromatography. Lane 1-marker (66 kDa), lane 2-fraction containing Lipoxygenases activity (peak 4). (c) Elution profile of Soybean Lipoxygenase after CM Sephadex C-50 cation exchange column chromatography (elution with 25 mM MES+175 mM NaCl buffer). (d) SDS-PAGE profile of Soybean Lipoxygenase after CM Sephadex C-50 cation exchange column chromatography. Lane 1-molecular weight marker (from top: 115 kDa; 66 kDa; lane 2-11 purified Lipoxygenase in increasing concentration.