

## Curcumin inhibits lipoxygenase by binding to its central cavity: theoretical and X-ray evidence.

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**Abstract.** Many lipoxygenase inhibitors including curcumin are currently being studied for their anti-carcinogenic properties. Curcumin is a naturally occurring polyphenolic phytochemical isolated from the powdered rhizome of the plant *Curcuma longa* that possesses anti-inflammatory properties and inhibits cancer formation in mice. Recently it was shown that the soybean lipoxygenase L1 catalyzed the oxygenation of curcumin and that curcumin can act as a lipoxygenase substrate. In the current study, we investigate the fate of curcumin when used as a soybean lipoxygenase L3 substrate. By use of X-ray diffraction and mass spectrometry, we have found an unoccupied electron mass that appears to be a unusual degradation product of curcumin (4-hydroxyperoxy-2-metoxyphenol) located near the soybean L3 catalytic site. Understanding how curcumin inhibits lipoxygenase may help in the development of novel anti-cancer drugs used for treatment where lipoxygenases are involved.

### Introduction

Lipoxygenase enzymes can be found in a wide variety of plant and animal tissues. Lipoxygenases are enzymes that possess a non-heme iron serving as a catalytic center for the stereo- and regio-specific dioxygenation of select carbon atoms in polyunsaturated fatty acids containing a 1,4-pentadiene motif. Eighteen carbon chain fatty acids (eg. linoleate) are the primary substrates of the plant lipoxygenases while the mammalian isozymes mainly catalyze the metabolism of fatty acids of carbon length 20 (eg. arachidonate). The soybean lipoxygenases were the first to be characterized and are named sequentially beginning with soybean

lipoxygenase 1 (L1), which was also the first lipoxygenase isozyme to have its three dimensional X-ray crystal structure solved [1, 2]. Nomenclature for the lipoxygenases found in mammals arises from the positional oxygenation along the carbon chain of arachidonic acid (AA). Mammalian lipoxygenases (LOXs) characterized thus far include 5-, 8-, 12-, and 15- type LOXs. Modeling of human lipoxygenases using pair-wise sequence identity has been performed previously [3] and several theoretical models of substrate mechanisms of action were discussed. To date, rabbit reticulocyte 15-LOX is the only mammalian LOX for which the three-dimensional X-ray structure has been obtained [4].

The amino acid sequences between plant and mammalian LOX enzymes show considerable homology. The soybean lipoxygenases, L1 [1] and L3 [5], are 72% identical in their amino acid sequences, but share only 25% sequence homology to any mammalian 15-LOX. Overall, sequence identity between plant and mammalian pairs of lipoxygenase isozymes is 21-27%, while plant pair sequence identity ranges from 43-86%, with mammalian pair sequences at 39-93% identity [3]. The highest level of sequence identity between lipoxygenases from plants and mammals lies in the area of the catalytic domain containing the non-heme iron atom. Mammalian lipoxygenases are 165-261 residues shorter than the plant lipoxygenases and were believed to lack a N-terminal  $\beta$  barrel due to the fact that similarities in the sequence identity of the first 200 residues between pairs of plants and animals never exceeds 15%. Comparisons between various mammalian lipoxygenase cDNAs have recently been reviewed [6]. The similarities in sequence data across species lead to the assumption of similar 3 dimensional structures and the comparison of soybean L3 with rabbit 15-LOX confirms that plant and mammalian enzymes share the same topology and overall architecture despite differences in size (Fig. 1).

Soybean lipoxygenases play physiological roles in processes such as growth, development, wound healing and senescence. As the main substrate for soybean lipoxygenases, linoleic acid is

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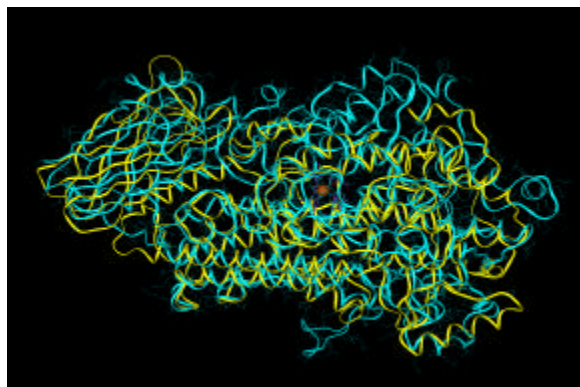


Fig 1. A Comparison of soybean L3 (blue) and rabbit 15-LOX (yellow) 3 dimensional structures. Iron is shown as a brown sphere. Please note the close similarities in topology despite the differences in size (L3: 857 residues; rabbit 15-LOX: 663 residues).

metabolized into one of various hydroperoxyoctadecadienoic acids (HPODEs). The biological significance of the HPODEs has yet to be fully characterized. Mammalian LOXs use arachidonic acid as the primary substrate which, once released from the mammalian membrane through the action of PLA<sub>2</sub> or a combination of other phospholipases [7], can be metabolized into leukotrienes (LT), lipoxins, or into eicosanoids. Leukotrienes and 5-HETE (hydroxyeicosatetraenoic acid) produced via the 5-LOX pathway have been shown to be active in promoting asthma and allergic airway inflammation [8]. Inhibitors of the 5-LOX pathway have chemopreventive abilities in animal lung carcinogenesis [9, 10] and block the oxidation of several potent carcinogens [11]. 5-HETE has the ability to stimulate growth of lung cancer cells [12], prostate cancer cells [13], and 5-LOX inhibitors have the ability to decrease cell proliferation and trigger apoptosis [14]. 12(S)-HETE, the major metabolite of the 12-LOX pathway, has been shown to correlate with metastatic potential [15] and stimulate the expression of integrin receptors leading to increased tumor cell adhesion [16]. Also, 12(S)-HETE can activate PKC, which mediates the secretion of cathepsin B, a cysteine protease that has been shown to be involved in tumor metastasis and invasion of colon cancer cells [17]. In prostate cancer patients, elevated 12-LOX mRNA levels were shown to correlate with poor differentiation and cancer cell invasiveness [18]. Additionally, 12-LOX in human prostate carcinoma stimulates angiogenesis and tumor growth [19]. Recently it was shown that 5-HETE and 12(S)-HETE directly stimulate pancreatic cell proliferation and that LOX inhibitors can induce apoptosis and cell differentiation [20]. 15-LOX and its products have been linked to cell maturation and differentiation [21] as well as implicated in several

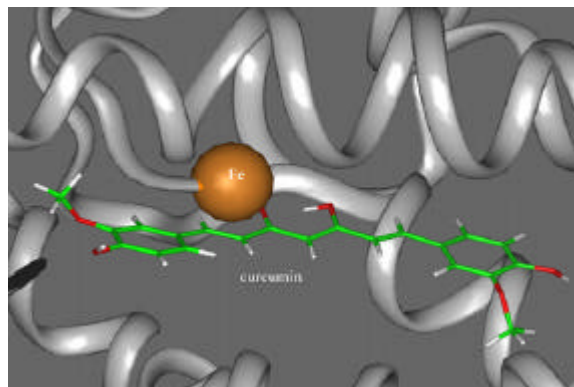


Fig. 2. Curcumin shown in the central cavity of soybean L3. Iron is shown in brown color, carbon in green, oxygen in red, hydrogen in white. L3 is shown as a ribbon model with the front part removed from the figure for clarity (theoretical predictions based on force field calculations).

aspects of atherosclerosis [22, 23]. As a whole, mammalian LOXs and products produced by substrate metabolism play significant roles in cancer cell growth, metastasis, invasiveness, and cell survival.

Many lipoxygenase inhibitors are currently being studied for their anti-carcinogenic properties. Curcumin is a naturally occurring polyphenolic phytochemical isolated from the powdered rhizome of the plant *Curcuma longa*. Curcumin has long been known to possess anti-inflammatory properties and is a commonly used spice in Asia. It has more recently been reported to inhibit tumorigenesis in mice [24]. Further, curcumin has the ability to decrease the formation of 5(S)-, 8(S)-, 12(S)-, and 15(S)-HETE in mouse epidermis [25].

Here we investigate the fate of curcumin with soybean lipoxygenase L3. Another group has studied the soybean L1 catalyzed oxygenation of curcumin [26] and shown that curcumin can act as a lipoxygenase substrate. Their data and the data presented herein, using soybean L3, lead to the assumption that curcumin can inhibit lipoxygenase activity by blocking the active site. By use of X-ray diffraction and mass spectrometry, we have found an electron mass located near the soybean L3 catalytic site. This mass appears to be an unusual degradation product of curcumin. Understanding how curcumin interacts with soybean L3 may explain how curcumin inhibits lipoxygenases and HETE formation. Due to the lack of structural data for human LOXs, researchers are still modeling human LOXs using soybean enzymes because of their availability and highly characterized structures. Use of plant lipoxygenases to model mammalian LOXs will prove highly beneficial and aid in structural characterization, mechanism elucidation, and possibly the discovery of novel inhibitors of LOXs.

## Materials and Methods

**Obtaining the Starting Structure.** All molecular modeling and structure visualizations were done on a SGI workstation using the *InsightII* program package from MSI [27]. Atomic coordinates of soybean L3 were as deposited in the Protein Data Bank (PDB entry 1LNH). Hydrogen atoms were added with appropriate charges assigned throughout the molecule of lipoxygenase assuming physiological pH 7.4. Partial and formal charges were assigned accordingly to the extensible systemic force field (esff).

**Docking.** The docking module enables the calculation of non-bonded energy between molecules assuming that fragments of the molecules are flexible. This program uses a score (*Ludi*) to quantify ligand-receptor binding for a fully energy minimized structure. The following parameters were used: radius of subset from Fe atom = 12 Å, maximum R change = 6 Å, maximum number of structures minimized = 7 (this number was usually higher since many different initial structures produced the same minimized structure), minimum steps = 100 or less if the maximum derivative was smaller than 0.01 kcal/mol/Å, MC temperature = 20, energy tolerance = 5000. Following this step, all structures were subject to *simulated annealing* where the temperature was raised to 500K then gradually reduced to 300K, after which 5 structures with a minimum potential energy were subjected to *molecular dynamics* for 1000 picoseconds. The structure with the minimum potential energy was accepted as the most probable one.

The *Ludi* scoring method of interactions between a protein and its ligand was used to quantify the binding characteristics of curcumin to lipoxygenase. The *Ludi* method for *de novo* design of ligands for proteins (*i. e.* enzyme inhibitors) is a method for screening a large number of compounds by analyzing the geometrical fit of given chemicals in the designated protein binding site. Other determinants of good binding are also calculated and include hydrogen bond formation, lipophilic interactions, ionic interactions, and acyclic interactions. However, *Ludi* can also score protein ligand interactions by statistically evaluating the fit of all potential ligands determined by the *Docking* module.  $Ludi \text{ Score} = -73.33 \text{ mol/kcal } \Delta G$ , where:  $\Delta G = \Delta G_o + \Delta G_{hb}f(\Delta R)f(\Delta\alpha) + \Delta G_{ion}f(\Delta R)f(\Delta\alpha) + \Delta G_{lipo}A_{lipo} + \Delta G_{rot}NR \Delta G$ ;  $\Delta G_o$  represents the contribution to the binding energy that does not directly depend on any specific interactions with the receptor (*i. e.* the contribution to binding energy due to loss of transitional and rotational entropy of the

fragment),  $\Delta G_{hb}$  and  $\Delta G_{ion}$  represent the contribution from an ideal hydrogen bond and unperturbed ionic interactions respectively,  $\Delta G_{lipo}$  represents the contribution from lipophilic interactions which is proportional to the lipophilic surface  $A_{lipo}$ ,  $\Delta G_{rot}$  represents the contribution due to freezing of internal degrees of freedom in the fragment, NR is the number of acyclic bonds,  $\Delta R$  is the deviation of the hydrogen bond length from the ideal value of 1.9 Å,  $\Delta\alpha$  is the deviation of the hydrogen bond angle from the ideal value of 180°. In general, a higher *Ludi* score (0-1100 in range) represents higher affinity and stronger binding of a ligand to the receptor

In addition, the *Ludi* score can be related to the dissociation constant  $K_i$ .  $Ludi \text{ Score} = -100 \log K_i$ .

**Isolation of soybean lipoxygenase (cutlivare Beeson 80)** was done as previously described [5]. Fractions from a chromatofocusing column were collected using a Gradi-Frac machine (Pharmacia Biotech; Piscataway, NJ) and the appropriate peak was concentrated using Centricon concentrators (Millipore; Bedford, MA). The concentrated protein solution was dialyzed against Tris buffer (pH 7.0) to remove the histidine buffer and was purified to a single band by SDS-PAGE.

**Crystallization and data collection.** Protein crystals were grown using the "sitting drop" method as described before [5]. Curcumin was dissolved in ethanol and added to the crystallization dishes so the final concentration of protein to curcumin was approximately 1:1 with ethanol <2%(v/v). The crystals became pale yellow and data was collected at room temperature using a RAXIS IV imaging plate detector with a Cu rotating anode and focusing mirrors. Crystal-to-detector distance was set at 140 mm with an exposure time of 12 minutes per frame and 2° oscillation. To avoid as much bias as possible, crystals of approximately the same size (~0.5mm) and shape were used to test varying soaking times and total exposure. After 45-90min (depending on crystal size), the crystal exposed to X-rays had changed to a purple color. This change of color was especially easy to notice on crystals bigger than the diameter of the collimator (0.5mm), where only the portion of the crystal irradiated by the X-ray beam turned purple with the rest of the crystal remaining yellow. This phenomenon was not observed in the curcumin solution when exposed to X-ray or in the crystal (no curcumin) when exposed to ambient light. The crystal soaked in curcumin remained purple for several hours following X-ray exposure and was still a light pink 24 hours

following. The reflections corresponding to the "purple" phase were processed and integrated using *Denzo* and *Scalepack* [28] resulting in three data sets corresponding to 15, 48, and 70 hours of soaking time with not more than 6 hours of total X-ray exposure per crystal. The data extended to a 2.1–2.2 Å resolution with 92–95% completeness and 6–8% of  $R_{merge}$  per set.

**Mass spectroscopy.** L3 crystals soaked with curcumin not exposed to X-ray and crystals after exposure to X-ray were dissolved in deionized water and analyzed by mass spectrometry at the Protein Structure Facility, University of Michigan, Ann Arbor, Michigan. Electrospray Ionization (ESI) mass spectroscopy was done using the VG Fisons "Platform" single quadrupole mass spectrometer ( $m/z$  limit 0–3,000). Samples were introduced into the mass spectrometer as  $H_2O$  solutions by flow injection at 5 microliters/min. Samples were examined in positive ion mode to look for protonated ions in the 0–3000 $m/z$  region, which was later electronically refined to give an expanded region of 0–500 $m/z$ . It is important to emphasize that in some cases molecules carry positive charges even without obvious chargeable sites, possibly because the charging can be affected by gas phase proton affinities [29–32].

## Results and Discussion

**Molecular simulations.** The size of the soybean L3 molecule exceeds the dimensions of the program for molecular simulations; therefore, the protein was restricted to radius of 20 Å around the proteins non-heme iron. This volume contains several channels enabling movement of the curcumin into the central cavity. Our calculations show that curcumin can bind to lipoxygenase in the central cavity close to the iron (Fig 2). The calculated affinity of curcumin for L3 reaches the high value of  $1.06 \times 10^{-10}M$ . This finding supports the hypothesis that the anticancer activity of curcumin could be linked to lipoxygenase inhibition. Dietary curcumin consumption levels can be high with no known toxic effects, but due to low water solubility only a fraction can enter the circulation. The high affinity of curcumin for L3, as calculated in the *Ludi* module, indicates that it possible to inhibit lipoxygenase with a low concentration of curcumin. To verify our theoretical findings we used X-ray crystallographic analyses.

**Soybean lipoxygenase purification.** Soybeans can be purchased in large quantities, thus providing a steady supply of protein and easily reproducible crystals for X-ray analysis. Two fractions from the top of the

soybean L3 peak were collected and purified (Fig 3) as described in Materials and Methods.

**Crystallography of the lipoxygenase-curcumin complex.** A change of color of lipoxygenase solutions has been previously observed and described [33]. It has been observed in lipoxygenase crystals in the presence of peroxides, such as 13-hydroperoxy-9,11-octadecadienoic acid (13HPOD) and cumene hydroperoxide [34]. The same phenomenon was noticed (i.e. the color change to blue/purple) with the iron complex  $[Fe(bppa)(t-BuCOO)]^{2+}$  when its *t*-butyl carboxylic acid ligand is substituted with *t*-butyl- or cumene hydroperoxide [35]. This color change is associated with the formation of an unstable complex, wherein the peroxy ligand is bound to the iron atom. Our experiments provide evidence that such complexes can exist longer, from several hours to a few days, when "trapped" in a crystal. In the case of the curcumin-L3 complex, the change of color was not observed in the crystals not illuminated by X-rays. This leads to the assumption that a photodynamic reaction is necessary for the change of color to occur. It is known; however, that curcumin can be photobleached, especially by shorter wavelengths of the light [36, 37]. The radical change in the crystals color, from yellow to purple, occurs only under X-ray illumination in both ambient light and in the dark. This leaves no doubt about the photodynamic characteristics of the observed phenomenon. Also, since the reaction takes place in the crystal, it is obvious that the curcumin molecule would have to be near the iron atom and in such an orientation that allows binding between the iron and the created peroxide.

The crystal unit cell was isomorphous monoclinic C2, ( $a=112.8$ ,  $b=137.3$ ,  $c=61.9\text{Å}$ ,  $\beta=95.5^\circ$ ) with that of the wild enzyme ( $a=112.8$ ,  $b=137.4$ ,  $c=61.9\text{Å}$ ,  $\beta=95.6^\circ$ ). The structure of this complex was solved by molecular replacement using native L3 as the starting model (PDB entry ILNH with subtracted  $H_2O$  molecules). The electron density maps (resolution 8–2.2Å, 36325 reflections,  $R=24\%$  with only protein atoms included) clearly show an unoccupied electron density near the iron atom. This mass is much too large to be a solvent molecule but smaller than curcumin. The shape and location of the mass do not agree with the forcefield calculated position of curcumin that underwent photodegradation during exposure to X-rays (Fig 4). The shape and volume of the unoccupied electron density in the immediate vicinity of the iron suggests the presence of a peroxide (Fig.5, molecule a). Our map does not show any evidence of the "prostaglandin-like" molecule (Fig. 5, molecule f)

which was found as a product of curcumin oxidation catalyzed by soybean L1 [26].

**Mass spectroscopy.** As expected, the mass spectrogram revealed numerous peaks that can be attributed to chemicals present in the media used in the crystallization of L3. However, at least four fragments with masses of 140, 157, 176, and 198 appeared on the spectrum from X-ray irradiated crystals but are absent on the spectrogram obtained by crystals not exposed to X-rays. These peaks could

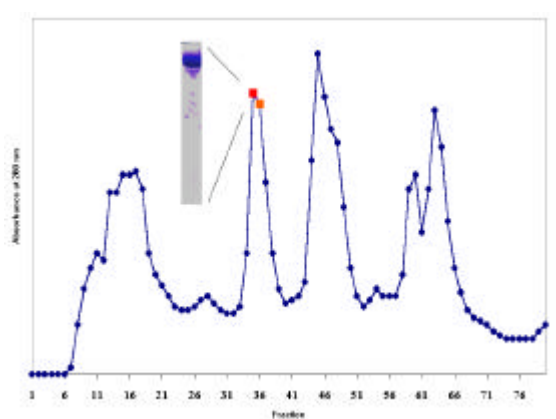


Fig. 3. The absorbance of different fractions during the purification of soybean L3. Only fractions indicated by squares on the graph were used for crystallization. The insert to the left of the peak is PAGE of this fraction, purified to >95%.

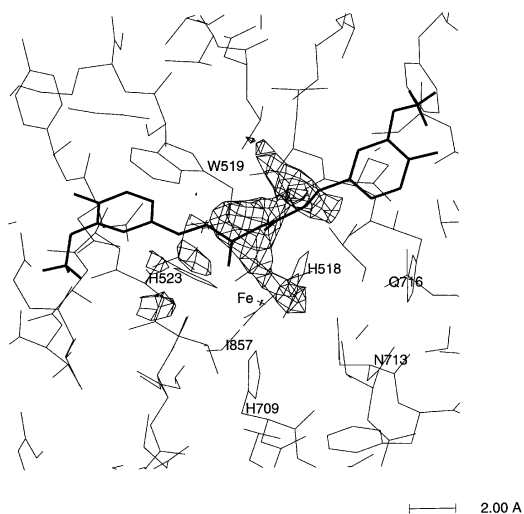


Fig. 4. L3 molecule with the observed unoccupied electron density (mesh) near the iron atom and a curcumin molecule (bold) in the position predicted by the force field calculations. The size, volume and position of the unoccupied ( $F_o - F_c$ ) difference map indicates the presence of the photodegradation product of curcumin corresponding to molecule 'a' in Fig.5.

be identified as potential products of the photodegradation of curcumin and are presented in Fig 5. The strongest candidate is compound 5a, a peroxide that, while unstable in complex with L3, can easily convert to compound 5b. Signals of 140 and 157 present on the mass spectrogram strongly support this assumption. If curcumin were oxygenated leading to the "prostaglandin-like" product (Fig. 5, molecule f), it would be present in both spectra, due to the fact that the experiment was conducted in the presence of oxygen and not in anaerobic conditions. Lack of any outstanding peak in this region (~400) indicates that either L3 does not react as L1 or, more likely, that such a molecule cannot be formed under the current experimental conditions without supplemental oxygen. The identity of all other compounds (Fig. 5, molecules c, d, and e) cannot be determined with absolute certainty and are outside scope of this paper.

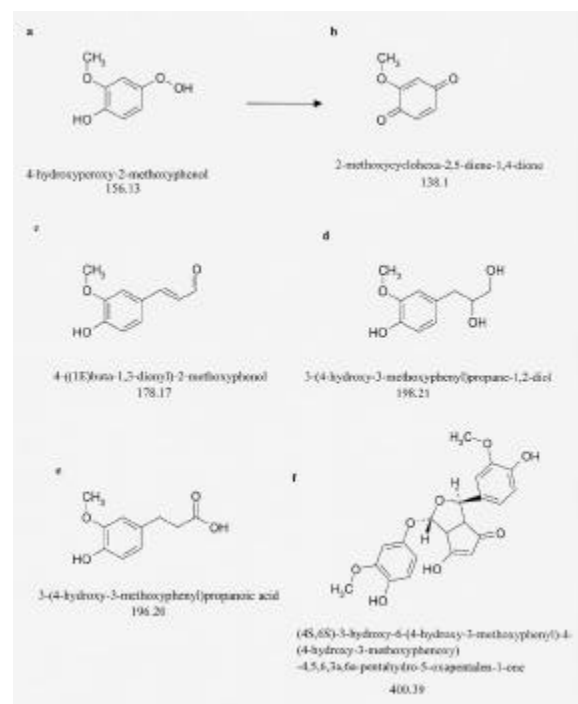


Fig. 5. Possible products of curcumin photodegradation (a-e) and a suggested product of curcumin oxidation catalyzed by soybean L1 (f).

The structural details of the complex between the photoproduct of curcumin degradation and L3 will be published upon completion of the crystallographic refinement. Several conclusions; however, are quite clear and can be included herein: (1) curcumin can penetrate L3 and bind in the central

cavity near the iron atom, (2) force field calculations predict a very low dissociation constant of  $10^{-10}$  M suggesting an very high affinity of curcumin as a lipoxygenase ligand making it a good candidate for an agonist/antagonist in drug design, (3) curcumin does not undergo L3 catalyzed oxidation and the complex is stable under normal conditions; although, (4) X-ray irradiation elicits a photodynamic reaction leading to the degradation of curcumin and the formation of a metastable complex consisting of L3 and a peroxy photoproduct, and (5) a certain period of time is necessary for the photodynamic reaction to produce enough peroxide molecules to react with L3

and for the color change characteristic of the complex to occur.

We hope that these observations may prove beneficial in the treatment and/or prevention of ailments where lipoxygenases are involved.

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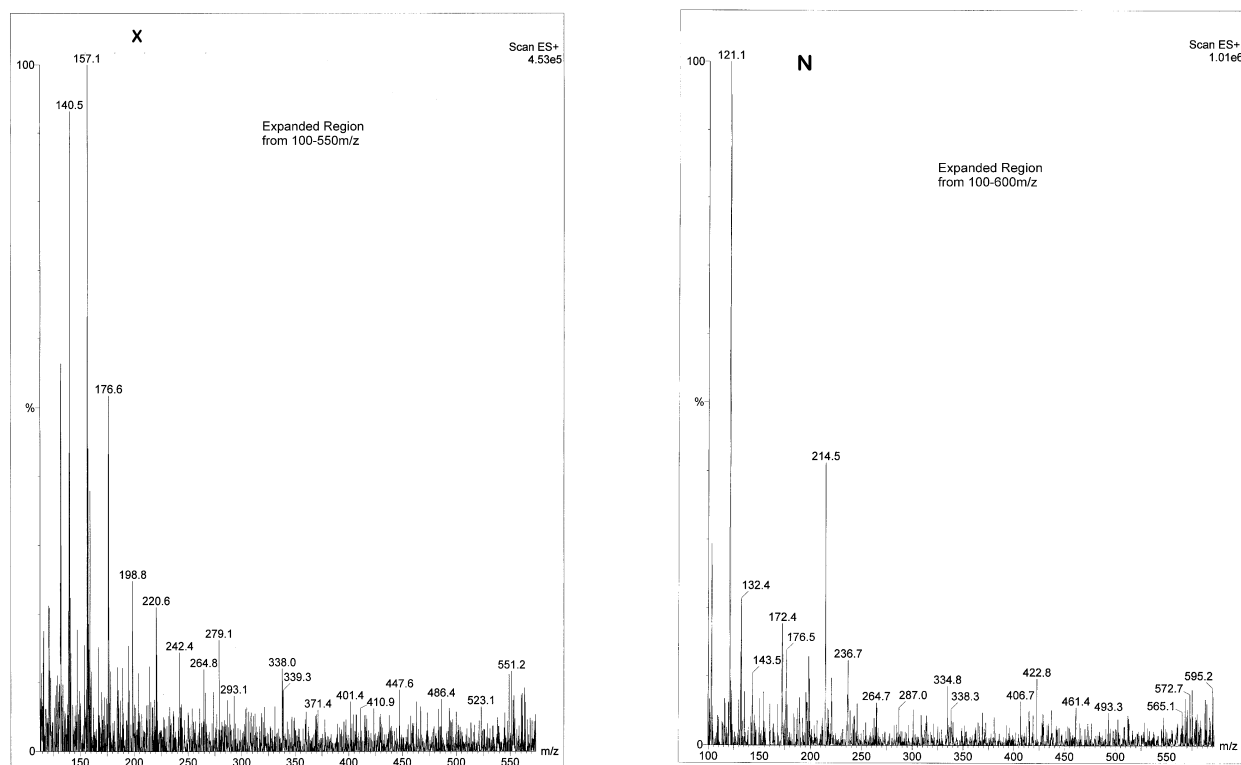


Fig 6. Mass spectra of soybean L3 crystals soaked with curcumin, not illuminated (N) and illuminated (X) by X-rays during data collection (expanded region from 100-500m/z).

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