Title: LIPOXYGENASE ENZYME ASSAY

\[ \text{Linoleic Acid} + O_2 \rightarrow 15-LO \]

\[ \text{13-HpODE}^* \]

\[ \text{Resorufin} \]

\[ \text{MicroPeroxidase} \]

\[ \text{Amplex UltraRed} \]

13-HydroPeroxyOctadecaDiEinoicacid

(57) Abstract: A method for identifying inhibitors of a lipoxygenase enzyme, the assay comprising: contacting a lipoxygenase enzyme with a test Compound, a lipoxygenase enzyme Substrate and oxygen; adding a fluorometric reagent and a peroxidase; measuring the fluorescent signal; determining the amount of enzyme inhibition by the test Compound.
Lipoxygenase Enzyme Assay

BACKGROUND OF THE INVENTION

APPLICATION DATA

This application claims benefit to US provisional application serial no. 60/820,390 filed July 26, 2006.

1. TECHNICAL FIELD

This invention relates to methods and kits for lipoxygenase enzymes which catalyze the oxygen-dependent oxidation of fatty acid substrates (linoleic acid and arachidonic acid are common examples) to form hydroperoxy-fatty acid products. The methods and kits are useful for detecting inhibitors of such enzymes.

2. BACKGROUND INFORMATION

Lipoxygenase enzymes catalyze the oxygen-dependent oxidation of fatty acid substrates (linoleic acid and arachidonic acid are common examples) to form hydroperoxy-fatty acid products. Enzymes have been purified from diverse organisms that display a broad range of substrate specificity and product specificity (i.e. the site of oxidation within the fatty acid).

Several assay procedures have been published in the literature but each has particular limitations that make high-throughput screening difficult. The simplest assay is the spectrophotometric monitoring of the hydroperoxy-fatty acid product; the hydroperoxy-moiety absorbs light at 234 nm and can therefore be easily monitored with a spectrophotometer. As many potential inhibitors absorb light at this wavelength, this assay format is prone to interference from the very compounds we seek. Another method of assaying for lipoxygenase activity is to monitor the consumption of oxygen using a Clark electrode; this method is neither sensitive nor amenable to high-throughput. Another assay that has been used is to determine the concentration of hydroperoxy (or the chemically-reduced hydroxy-derivatives) fatty acids by separation from the substrate on a high-performance liquid chromatography (HPLC) system (for example, Yamamoto et al. (1990) Methods in Enzymology, 186, 371-380). These assays, while accurate, are heterogeneous and time consuming and are therefore not
amenable to screening large numbers of compounds. Two different colorimetric assay formats have been developed that utilize the oxidation state of the hydroperoxy product to couple product formation to color formation. Both assays are conducted in two steps and differ on the colorimetric reagent. After product has been formed, the color-forming reagent is added and the color is measured on a spectrophotometer. One assay used a xylene orange:iron(II) complex (Waslidge et al. (1995) Anal. Biochemistry, 231, 354-358) and the second assay used hemoglobin as the catalyst) and N-benzoyl leucomethylene (Auerbach et al. (1992) Anal. Biochemistry, 201, 375-380) as the colorimetric reagent. These assays offer improved sensitivity over the direct spectrophotometric assay (~10-fold) and improved throughput when compared to the HPLC method. However, colorimetric assays suffer from a small signal-to-background window in which to measure a signal. Kratky et al. have published a very sensitive assay of lipoxygenases based upon chemiluminescent detection (Kratky et al. (1999) Biochimica et Biophysica Acta, 1437, 13-22). The hydroperoxy-fatty acid product of lipoxygenase is reacted with isoluminol and microperoxidase to form an electronically excited form of 4-aminophthalate that emits a photon upon its decay. Because chemiluminescence is very short lived, each individual assay must be initiated and completed before proceeding to the next assay. This process makes the assay unsuitable for a high-throughput approach.

Molecular Probes (now part of Invitrogen) has published an assay for hydrogen peroxide detection that employs Amplex Red® (N-acetyl-3,7-dihydroxyphenoxazine) or Amplex UltraRed® and uses horseradish peroxidase as the redox catalyst instead of microperoxidase (Zhou et al. (1997) Anal. Biochemistry, 253, 162-168). While they sell many kits based upon the ability to couple hydrogen peroxide with Ample Red® oxidation, they do not mention the ability use the Amplex Red® reagent to detect hydroperoxy-fatty acids nor do any of their present reagents use microperoxidase as the redox catalyst.

The present inventors have designed an assay format to enable the identification of inhibitors of lipoxygenase enzymes. This assay represents a significant advantage over previous assay formats as the sensitivity and uniqueness of the signal render the format more amenable to high-throughput screening.
BRIEF SUMMARY OF THE INVENTION

It is therefore an object of the invention to provide a method to identify inhibitor of lipoxygenase enzymes.

It is a further object of the invention to provide a kit comprising an assay to identify inhibitor of lipoxygenase enzymes.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic depiction of microperoxidase catalyzing a redox reaction between the hydroperoxy-fatty acid product and the Amplex UltraRed® to generate the highly fluorescent product resorufin. The amount of resorufin is then determined using fluorescence spectroscopy.

Figure 2: Schematic depiction of the fluorometric lipoxygenase assay that would be used to characterize the activity of a 15-lipoxygenase.

Figures 3-5: Inhibition of x-lipoxygenase by representative compounds of varying potencies. The y-axis is Percent of Control and the x-axis units are in microM.

DETAILED DESCRIPTION OF THE INVENTION

To increase assay sensitivity and retain high-throughput features (homogenous assay that can be easily automated), the present invention provides a new assay for lipoxygenase which is an improvement from the historical assays described above.

After the lipoxygenase has been incubated with the fatty acid substrate (linoleic acid or arachidonic acid) and oxygen, microperoxidase (a catalyst) and Amplex UltraRed® are added. The microperoxidase catalyzes a redox reaction between the hydroperoxy-fatty acid product and the Amplex UltraRed® to generate the highly fluorescent product resorufin. The amount of resorufin is then determined using fluorescence spectroscopy (excitation at 530 nm and emission at 580 nm). See figure 1. This assay improves the sensitivity ~10-fold over that observed in the colorimetric assays and generates a
fluorescent signal that is both stable and free from compound interference as very few compounds fluoresce in this range.

In the broadest generic embodiment, there is provided a method for identifying inhibitors of lipoxygenase enzymes, the assay comprising:

contacting a lipoxygenase enzyme with a test compound, a lipoxygenase enzyme substrate and oxygen;

adding a fluorometric reagent and a peroxidase;

measuring the fluorescent signal;

determining the amount of enzyme inhibition by the test compound.

In second generic embodiment, there is provided a kit for determining the amount of lipoxygenase enzyme inhibition by a test compound comprising:

a lipoxygenase enzyme;

a lipoxygenase enzyme substrate;

oxygen;

a peroxidase and a fluorometric reagent.

The above kit can further contain a positive control that comprises a mock test compound. Said mock test compound having no or negligible lipoxygenase enzyme inhibition.

The Enzymes have been purified from diverse organisms that display a broad range of substrate specificity and product specificity. The assay as it is routinely performed is summarized in the scheme from the example section below but alterations apparent to those of ordinary skill in the art can be made. For instance, the incubation time or temperature can be adjusted but it is ideal to adjust them such that the enzyme activity is within the linear response range. The assay has been performed at various scales (cuvet, 96 or 384 well) and is expected to work at any scale required within any desired reaction vessel (e.g. polypropylene micro-plate or polystyrene cuvet). Any lipoxygenase enzyme that produces a hydroperoxy product, irrespective of stereo-specificity, is capable of being assayed by this technique, including 15-lipoxygenase from humans or soybean, 12-lipoxygenase and 5-lipoxygenase. Any substrate of the
lipoxygenase enzyme can be used; this could include, but is not limited to, free fatty acids or esterified fatty acids of varying composition (e.g. arachidonic acid, linoleyl-phosphatidyl choline, low-density lipoprotein, etc.). While Amplex UltraRed® is the preferred fluorometric reagent in this protocol, Amplex Red® or any reagent that results in the production of a fluorescent molecule with similar fluorescence (excitation maximum of 530 ± 25nm and emission maximum of 580 ± 25nm) can also be used. Similarly, microperoxidase may be substituted with any peroxidase that catalyzes the reaction between the hydroperoxide product and the fluorometric reagent (i.e. Amplex UltraRed® in the preferred embodiment). The solutions used for the lipoxygenase reaction and the microperoxidase reaction may also be modified from the specified conditions so long as activity of the lipoxygenase and microperoxidase catalysts are retained. Examples of the use of this assay identify lipoxygenase inhibitors are shown in figures 3-5.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

The Assay according to the invention can be performed according to the scheme shown in figure 2.

All referenced cited in this application are incorporated herein by reference in their entirety.
What is Claimed is:

1. A method for identifying inhibitors of a lipoxygenase enzyme, the assay comprising:
contacting a lipoxygenase enzyme with a test compound, a lipoxygenase enzyme substrate and oxygen;
adding a fluorometric reagent and a peroxidase;
measuring the fluorescent signal;
determining the amount of enzyme inhibition by the test compound.

2. The method according to claim 1 wherein the lipoxygenase enzymes are chosen from 15-lipoxygenase, 12-lipoxygenase and 5-lipoxygenase.

3. The method according to claim 2 wherein
the substrate of the lipoxygenase enzyme is a free fatty acid or esterified fatty acid.

4. The method according to claim 3 wherein
the fluorometric reagent is a reagent that results in the production of a fluorescent molecule with an excitation maximum of 530 ± 25nm and emission maximum of 580 ± 25nm.

5. The method according to claim 4 wherein
the fluorometric reagent is Amplex UltraRed® and the peroxidase is microperoxidase.

6. A kit for determining the amount of lipoxygenase enzyme inhibition by a test compound comprising:
a lipoxygenase enzyme;
a lipoxygenase enzyme substrate;
oxygen;
a peroxidase and a fluorometric reagent.

7. The kit according to claim 6 further comprising a positive control.
FIG. 1/5

Linoleic Acid + O₂ → 15-LO → 13-HpODE* → Resorufin Fl 530/580nm

MicroPeroxidase

Amplex®
UltraRed

*13-HydroPeroxyOctadecaDiEnoic acid

13-HODE
**Lipoygenase Reaction**
50 μl (96well) or 15 μl (384well)
0.1 M Tris (pH 8), 0.1 % BSA
20 nM Lipoygenase
125 μM Linoleic Acid
20 μM Amplex® UltraRed

10 minutes at room temperature

**Microperoxidase Reaction**
Add 50 μl (96well) or 15 μl (384well)
1.6 μM Microperoxidase
0.5 M CAPS (pH 10)

10 minutes at room temperature

**Measure Fluorescence**
Excitation=530nm
Emission=580nm
FIG. 3/5
**A. CLASSIFICATION OF SUBJECT MATTER**

INV. C12Q1/26

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>FR 2 520 006 A (TOYO JOZO KK [JP]) 22 July 1983 (1983-07-22) page 6, lines 14-22; claims 1,6,9</td>
<td>6,7</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

*A*: document defining the general state of the art which is not considered to be of particular relevance

*E*: earlier document but published on or after the international filing date

*L*: document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

*C*: document referring to an oral disclosure, issue, exhibition or other means

*P*: document published prior to the international filing date but later than the priority date claimed

*": later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

*X": document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y": document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

*": document member of the same patent family

Date of the actual commencement of the international search: 12 November 2007

Date of mailing of the international search report: 20/11/2007

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 Hl Alphen
Tel: (+31-70) 340-2040, Tx: 31 651 epo nl, Fax: (+31-70) 340-3016

Authorized officer:

Pellegrini, Paolo
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document with indication where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>FR 2520006 A</td>
<td>22-07-1983</td>
<td>DE 3301655 A1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 58126798 A</td>
</tr>
</tbody>
</table>