USE OF EXTRACTS CLUSTER ANALYSIS (ExCLAN) TO RAPIDLY SCREEN FOR ENZYME INHIBITORS IN MICROBIAL FERMENTED EXTRACTS
Vasantha KY, Murugesh CS and Sattur AP*
Fermentation Technology and Bioengineering Department, Central Food Technological Research Institute, Mysore 500020, India

Received for publication: February 02, 2014; Revised: February 11, 2014; Accepted: March 21, 2014

Abstract: The success of a new molecule discovery programme lies in the effectiveness of the primary screen for desired activities. A rapid screen, Extracts Cluster Analysis (ExCLAN) was used for the screening of lipoxygenase inhibitors from microbial extracts. About 50 crude extracts were randomly grouped as 5 clusters and screened for inhibition. Cluster C3, which showed 93% inhibition, was further deconvoluted, to pinpoint isolate P25 as the extract with the inhibitor. Further isolation and purification identified the inhibitor as sclerotiorin with an IC₅₀ value of 4.6µM against soybean lipoxygenase. This method helped in significantly reducing the time and number of assays with subsequent savings in costs of conducting the assays.

Keywords: Lipoxygenase Inhibitors, Microbial Extracts, Cluster Analysis, Screening, Sclerotiorin, Nigerloxin

INTRODUCTION
Several steps are involved in the screening process of natural products, with the primary or initial screen, being the most critical part of the entire programme. The primary screening process is essential in estimating both high hit rates and provide for large number of samples to be screened rapidly. This also enables the use of either semi or fully automated systems referred as HTS, wherein large number of samples can be screened with minimum number of steps. The greatest challenge in the primary screening is to subject large numbers of extracts to a faster screening process with high efficiency and low costs per sample, to pinpoint the desired activity. [1] There are a few methods reported for rapid biological screening, but they are restricted to screening synthetically produced structures. [2,3,4] During the course of our work on discovery of novel lipoxygenase inhibitors from microbes, we found it necessary to establish a method which was relatively quick and with a reliable accuracy during the primary screening itself, as the existing methods of single sample-at-a-time screening was laborious, slow and expensive. Thus, the present manuscript deals with the use of a cluster approach as a fast and reliable primary screening technique, for pinpointing potential enzyme inhibitors from large number of extracts.

MATERIALS AND METHODS
Isolation of fungi from soil flora
About a hundred soil samples were collected from various places in Mysore, Karnataka, India. The soil samples were serially plated on to potato dextrose agar (PDA) screening medium and incubated at 28°C, upon growth individual colonies were transferred to other plates successively until the isolation of pure cultures.

Fermentation and extraction
Spore suspensions of all the isolated cultures from the 5 day old culture slant was prepared by adding 15 ml of sterile 0.1% Tween-20 (Himedia, India) and gently scraping the agar surface with a sterile loop, to liberate spores. One milliliter of this spore suspension were inoculated to Erlenmeyer flasks containing 100ml of potato dextrose broth (PDB), (Himedia, India) which was previously autoclaved at 121°C and 15 lbs. for 15 min. The inoculated flasks were kept for fermentation at 30°C on a rotary shaker operating at 200 rpm. After 7 days, to each fermented broth, EtOAc (1:1 v/v) was added and upon vacuum distillation. The crude extracts obtained were screened for lipoxygenase inhibition.

Isolation and purification of the inhibitor
The crude extract containing the inhibitor was chromatographed on a column (1 x 50 cm) packed with silica gel (60-120 mesh) and eluted with hexane (200 mL, 1 mL/min) to remove impurities followed by elution with 200 mL benzene.

Structure elucidation of the inhibitor
¹H NMR spectra were recorded at 500 MHz (500.13 MHz proton and 125 MHz 13C). A 30 mg of solid sample dissolved in CDCl₃ was used for recording the NMR spectra at 27°C. For GC /EIMS conditions, a BP-1 capillary column was used with temperature program as follows: 120°C at 10°C/min to 180°C at 4°C/min to 280°C (15 min); injection temperatures, 250°C; detector (flame ionization detection) temperature at 280°C; 2 ml/min flow rate (N₂).

*Corresponding Author:
Dr. Sattur AP,
Fermentation Technology and Bio engineering Department,
Central Food Technological Research Institute,
Mysore 500020, India.
**Lipoxygenase assay**

The assay was carried out by monitoring the appearance of cis, trans-hydroperoxide derivative at 234 nm. LOX-1 was partially purified from soybeans (Hardy variety) with a specific activity of 135 units/mg protein. The assay mixture contained 20μL of enzyme, 10-20 μL of crude extract or sclerotiorin (2 mg/mL DMSO stock), and 2.955 mL of 0.2 M sodium borate buffer (pH 9.0). The reaction was initiated by the addition of 15μL of substrate (10 mM linolenic acid). The control sample received equal volumes of DMSO without the inhibitor. The relative activity was expressed as the percentage of the enzyme activity in relation to the control without the inhibitor. [5].

**RESULTS AND DISCUSSION**

Screening of active compounds from extracts derived from natural sources such as bacteria, fungi of terrestrial or marine origin or plants is more difficult than synthetic compounds due to its complex chemical nature created by a number of (dis)similar compounds. Thus far, some creative methods have been developed for rapid inhibitor screening such as Frontal Affinity Chromatography coupled with Liquid Chromatography and Mass Spectrometry [6], Size Exclusion Chromatography coupled with ESI-LC-MS/MS, described by Sonal et al., [7] and use of enzyme metabolite reaction mixture with HPLC [8]. Other approaches for mixture screening have been reported, including Surface Plasmon Resonance, NMR and affinity selection-mass spectrometry method. [9]

**Primary Screening by Extracts Clusters Analysis (ExCLAN) Technique**

This method comprised four steps. The first one involved clustering of 50 crude extracts, wherein each cluster contained 10 crude extracts. These were randomly selected for a cluster and mixed such that the final absorbance at 234 nm did not exceed 0.6 OD. This was the critical step as strongly coloured compounds in the extracts could interfere in the assay. Further, careful selection of clusters at this stage could prevent false positives or negatives. Thus, 5 clusters of 100 μg each were made from 10 μg from each of the 50 extracts. (Figure 1) The second step was the initial detection of enzyme inhibition from the 5 clusters (10 μg each). So, C1 to C5 clusters gave inhibition assays A1 to A5, respectively. The cluster (C5) which showed 93% inhibition (A3 or Assay 3) was selected. (Table 1). In the third step the active cluster, C3, was deconvoluted randomly into three groups, in which one of them (A6) contained 4 crude extracts and the others (A7 and A8) had each three. The three groups were assayed again against Lipoxygenase enzyme and A7 gave the highest inhibition of 93% (Table 1). The final step was the identification of the specific crude extract with inhibition from the selected group. For this each crude extract of the selected group, A7 was subjected to the inhibition assay individually, which identified the crude extract P25 as the source of the inhibitor. (Table 1).

**Table 1: Detection of Lipoxygenase inhibitor by ExCLAN method**

<table>
<thead>
<tr>
<th>Cluster name (assay number)</th>
<th>% inhibition</th>
<th>Inhibition for the deconvoluted groups from C3</th>
<th>Cluster name, (assay number)</th>
<th>% inhibition</th>
<th>Isolate of crude extract (assay number)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cluster from the 5 clusters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1 (A1)</td>
<td>15.0 ± 0.26</td>
<td>C3 (A6)</td>
<td>NIL</td>
<td>P25 (A9)</td>
<td>89.0 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>C2 (A2)</td>
<td>Nil</td>
<td>C3 (A7)</td>
<td>90.8 ± 0.19</td>
<td>P26 (A10)</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>C3 (A3)</td>
<td>93.0 ± 0.17</td>
<td>C3 (A8)</td>
<td>5.0 ± 0.17</td>
<td>P27 (A11)</td>
<td>NIL</td>
<td></td>
</tr>
<tr>
<td>C4 (A4)</td>
<td>4.2 ± 0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5 (A5)</td>
<td>9.1 ± 0.40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*extracts of isolates P21, P22, P23, P24;*  
*b extracts of isolates P25, P26, P27;*  
*e extracts of isolates P28, P29, P30;*  

**Fig.1: Rapid screening of enzyme inhibitors by Extracts Cluster Analysis (ExCLAN) method**

In order to validate the primary screen method, Nigerloxin, a potent inhibitor from Aspergillus Niger MTCC 5116 discovered in our lab, was used as a standard lipoxygenase inhibitor. [10]. The inhibitor was incorporated in three clusters at the same concentration as that of the crude extracts in the clusters. The clusters which have did not show any inhibition in the previous assays were selected to avoid any false results. Hence, the clusters which gave inhibition with Nigerloxin incorporated into them was solely due to the presence of the inhibitor and the non-inhibitory effect of the same clusters without Nigerloxin shows that formation of clusters did not affect the activity of the crude extracts. (Table 2)
Table 2: Validation of ExCLAN using standard lipoxygenase inhibitor Nigerloxin

<table>
<thead>
<tr>
<th>Cluster name</th>
<th>%Inhibition *</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 (without Nigerloxin)</td>
<td>Nil</td>
</tr>
<tr>
<td>C2 (without Nigerloxin)</td>
<td>Nil</td>
</tr>
<tr>
<td>C4 (without Nigerloxin)</td>
<td>Nil</td>
</tr>
<tr>
<td>C1 (with Nigerloxin)</td>
<td>90.0 ± 0.23</td>
</tr>
<tr>
<td>C2 (with Nigerloxin)</td>
<td>85.2 ± 0.15</td>
</tr>
<tr>
<td>C4 (with Nigerloxin)</td>
<td>88.0 ± 0.08</td>
</tr>
</tbody>
</table>

*inhibition at 1 µg of each crude extract in 10 µL of cluster.

Isolation and purification of the inhibitor from extract of isolate P25

500 mg crude extract giving an inhibition of 93% was loaded on silica gel column and eluted with hexane (200 mL) to remove impurities. The elution solvent was then changed to benzene, collected into 3 fractions and distilled under vacuum. The inhibitory activities of these 3 fractions at 1mg mL⁻¹ were nil, 90% and 8% respectively. The characteristic spectroscopic data obtained for the inhibitor were UV spectrum (methanol, 0.5 mg/mL) λmax: 284 and 361 nm. The structure determination of the inhibitor in fraction 2 was done using NMR and GC MS.¹H NMR spectrum, ¹H NMR( 500 MHZ, CDCl₃): 7.94 (1H, s, H-1), 6.60 (1H, s, H-4), 6.08 (1H, d, J = 15.7 Hz, H-9), 7.06 (1H, d, J = 15.6 Hz, H-10), 5.71(1H, d, J = 9.7 Hz, H-12), 2.49 (1H, m, H-13), 1.34 (1H, m, H₂-14), 1.44 (1H,m, H₂-14), 0.87 (3H, t, J = 7.3 Hz, H-15), 1.01 (3H, d, J = 6.6 Hz, H-16), 1.85 (3H, s, H-17), 1.57 (3H, s, H-18), 2.17 (3H, s, H-20) and ¹³C NMR (125 MHZ, CDCl₃): 153.3 (C1), 158.8 (C2), 107.0 (C4), 139.3 (C5), 115.2 (C5), 192.4 (C6), 85.2 (C7), 186.6 (C8), 111.4 (C8a), 116.3 (C9), 143.5 (C10), 132.6 (C11), 149.5 (C12), 35.8 (C13), 30.7 (C14), 12.6 (C15), 20.8 (C16), 13.0 (C17), 23.2 (C18), 170.7 (COCH₃), 20.7 (COCH₃). From NMR and GC-MS spectrum the structure of the compound was determined as Sclerotiorin. (Figure 2). The IC₅₀ value of the compound was determined as 4.6 µM. (Figure 3) This was fairly in close range with the IC₅₀ value of the same Lipoxygenase inhibitor (4.2 µM) produced by Penicillium frequentans CFTRI A-24,[11].

CONCLUSION

In the course of a discovery programme on new molecules from natural sources, once a target enzyme is reported and its assay standardized, usually hundreds of extracts are screened for inhibitors. The success of the screen is dependent on the quality of hits and the costs of running the primary screen, but it is usually the costs that decides its popularity of usage. Hence, it is against this background that this method in this study was found to be reliable, cost effective and time saving as a primary screen. Further improvements to the ExCLAN method with respect to solvent compatibilities, homogenous mixing of crude extracts, avoidance of precipitates in clusters, scaling up of clusters to accommodate various types of assays are in progress.

ACKNOWLEDGEMENT

The funding for this work from the 11th Five Year Plan CSIR Network Project on “Exploitation of India’s Rich Microbial Diversity” is gratefully acknowledged.

REFERENCES


Source of support: 11th Five Year Plan CSIR Network Project on “Exploitation of India’s Rich Microbial Diversity”

Conflict of interest: None Declared