Biochemical and Biophysical Research Communications 453 (2014) 784–786

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Development of a bioautographic method for the detection of lipase inhibitors

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ARTICLE INFO

Article history:
Received 4 October 2014
Available online 14 October 2014

Keywords:
Autobiographic method
p-Nitrophenyl butyrate
Bromothymol blue
Lipase inhibitor
Orlistat
Streptomyces

ABSTRACT

An autobiographic method based on the thin layer chromatogram was developed by using the chemical system that comprises *p*-Nitrophenyl butyrate and bromothymol blue for detecting the lipase inhibitor. Lipase inhibitory zones were visualized as blue spots against the greenish yellow background. This method could able to detect the well known lipase inhibitor, orlistat up to the concentration of 1 ng which is better than the earlier method. This method could also able to detect the lipase inhibition activities from the un-explored species of *Streptomyces*. The developed method can be used not only for the screening of unknown samples for the lipase inhibitors but also for the purification of the lipase inhibitors from the unknown samples.

1. Introduction

Lipase (EC 3.1.1.3) is required for the digestion of triglycerides and inhibition of this enzyme reduces the absorption of fat and leads to the loss of body weight. Recent approaches for the treatment of obesity focused on the inhibition of dietary triglyceride absorption via pancreatic lipase inhibition [1,2]. Lipase inhibitors will also be helpful in treating atherosclerosis. Lipase inhibitors are mainly reported from plant and microbial sources [2]. However, till now orlistat is the only pancreatic lipase inhibitor that was approved clinically for the treatment of obesity [3]. But, recently orlistat was reported to show the adverse side effects like acute kidney injury and oxalate nephropathy [4]. Hence, there is a requirement for the lipase inhibitors that has no or reduced side effects.

Usually, people prefer therapeutic molecules from the natural resources over synthetic ones due to their reduced side effects. However, the screening of natural resources for lipase inhibitors is cumbersome and there is a need for the quick and effective method. The chromatography based autobiographic methods were reported to determine the activity *in situ* and quickly provide the information on the presence and localization of activity in the complex plant matrices [5]. Autobiographic methods were used for the detection of antifungal activity initially [6] and later used for the determination of acetylcholine esterase inhibitors [7,8] and glucosidase inhibitors [5]. Recently, TLC based bioautographic method was developed for the detection of lipase inhibitors from *Camellia sinensis and Rosmarinus officinalis* [9]. This method was developed by using naphthyl acetate and fast blue salt B. Recently, lipase inhibition activity was assayed spectrophotometrically by using *p*-Nitrophenyl butyrate as a substrate and this method is faster [10]. However, this substrate was not explored for the development of bioautographic method. In the present study, *p*-Nitrophenyl butyrate was used along with bromothymol blue, a pH indicator for the development of bioautographic method for the detection of lipase inhibitors. This method could able to localize the orlistat and this method was used to detect the lipase inhibitors from the cultures of un-explored species of *Streptomyces*.

2. Materials and methods

2.1. Microbial cultures and reagents

Cultures of *Streptomyces coelicolor*, *Streptomyces tendae*, *Streptomyces aurantiacus* and *Streptomyces albaduncus* were obtained from microbial type culture collection, Chandigarh, India and maintained on the agar slants containing the medium 1 (0.4% yeast extract, 1% malt extract and 0.4% glucose; pH 7.2) for *S. coelicolor* and medium 2 (0.4% glucose, 0.4% yeast extract, 0.4% meat extract and 0.2% $CaCO_3$; pH 7.2) for the other cultures. They were grown as submerged cultures in the medium containing glucose (0.5%), glycerol (2%), soya bean meal (2%), yeast extract (0.5%) and NaCl (0.3%) for the production of lipase inhibitor at 28 °C and 150 RPM for 5–7 days. Orlistat, porcine pancreatic lipase and

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http://dx.doi.org/10.1016/j.bbrc.2014.10.030
0006-291X/

p-Nitrophenyl butyrate (PNPB) were obtained from Sigma. Bromothymol blue and methanol were purchased from Nice Chemicals Pvt. Ltd., India. Chloroform and benzene were obtained from Spectrochem, India. Silica gel 60 F_{254} plates (Merck) were used for carrying out TLC.

2.2. Bioautographic assay

Orlistat was dissolved into water (pH 7.7) at the concentration of 120 mg/ml and 10 μl of appropriately diluted sample was used for the loading on the TLC plate. Cultures of *Streptomyces* were centrifuged after the growth and 20 μl of the supernatant was loaded on to the plate after adjusting the pH to 7.7. Solvent system containing chloroform and methanol (90:10) was used as mobile phase for the separation of orlistat and the one containing benzene and methanol (60:40) was used for the separation of supernatants of *Streptomyces* cultures. The TLC plate was air dried till the solvent was evaporated completely after the separation of samples. Then the plate was sprayed with the porcine pancreatic lipase enzyme dissolved (10 mg/ml) in the water (pH 7.7) and dried at room temperature. Later the plate was incubated in the humidified chamber at 37 °C for 1 h in order to allow the enzyme–inhibitor interaction to take place. Then the plate was air dried and sprayed with PNPB (200 μM) prepared in the water (pH 7.7). After air drying, the plate was incubated at 37 °C for 30 min for allowing the enzyme and substrate reaction to take place. Then the plate was air dried and sprayed with bromothymol blue solution to visualize the lipase inhibitor as blue spot against the greenish yellow background.

2.3. Lipase inhibition assay

The blue spot on the TLC plate was scrapped and suspended in the water sufficient to submerge the silica gel for 3–4 h and silica was separated by centrifuging for 3 min at 10,000 RPM. The supernatant was separated and extracted with equal volume of ethyl acetate by shaking at 150 rpm and 30 °C for 60 min. Then the mixture was centrifuged at 10,000 RPM for 30 min and the organic layer was collected and dried at 40 °C. Afterwards, the sample was dissolved into the minimum volume of DMSO and the 40 μl of it was used for assaying the lipase inhibition activity. Pancreatic lipase corresponding to the 20 units was pre-incubated with the extract of the blue spot for 60 min at 37 °C before adding the substrate. The percentage of residual activity of the lipase was determined by the spectrophotometric assay described below. Here, PNPB (200 μM) was employed as the lipase substrate and the reaction was maintained at 37 °C for 30 min and the release of *p*-Nitrophenol was measured at 410 nm. The enzyme activity was detected as the μ moles of *p*-Nitrophenol released per minute. Orlistat was used as a positive control in the assay and the % of enzyme inhibition was calculated with reference to the control that has solvent instead of extract [10].

3. Results and discussion

In order to screen un-explored species of *Streptomyces* for the lipase inhibitors an autobiographic method based on the TLC was developed. The principle behind this assay is lipase leads to the formation of *p*-Nitrophenol and butyric acid after acting upon *p*-Nitrophenyl butyrate and the released butyric acid will bring down the pH of the reaction mixture. Hence, the addition of pH indicator must indicate this change by means of color change. We have selected bromothymol blue since this indicator will be in blue color when the pH was above 7.6 and its color will be changed to light green in the pH range of 6.0–7.6. The pH of all

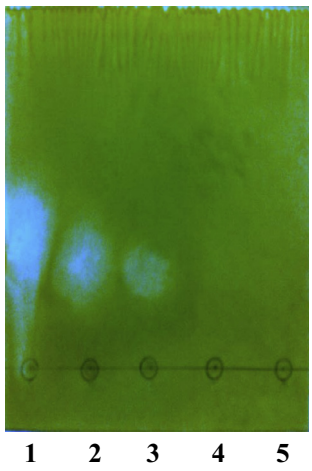

Fig. 1. Bioautographic thin layer chromatogram performed using different concentrations of orlistat. Lanes 1, 2, 3 and 4 spotted with 100, 10, 1 and 0.1 ng of orlistat, respectively. Lane 5 was spotted with distilled water.

the reagents used in this assay was adjusted to 7.7. Hence, wherever the lipase acts it must show up as green and the spots where there is no lipase action must be visualized as blue when the bromothymol blue was sprayed. To test this possibility on the TLC plate we have spotted the different concentrations of well known lipase inhibitor, the orlistat on the plate and samples were separated by using the solvent system that contains chloroform and methanol and the plate was developed by using lipase, PNPB and bromothymol blue as described in Section 2. After the bioautographic assay the presence of blue spots against the greenish yellow background was observed in the lanes of TLC plate (Fig. 1) spotted with orlistat. However blue spot was not appeared when 0.1 ng (Fig. 1, lane 4) of orlistat was used suggesting the limit of detection by this method and it could able to detect the orlistat up to 1 ng (Fig. 1, lane 3). The sensitivity of this method is better than the autobiographic assay based on the chemical system containing naphthyl acetate and fast blue salt B for the detection of orlistat [9]. Yellow color in the background is due to the *p*-Nitrophenol that formed after the enzymatic reaction. The R_f

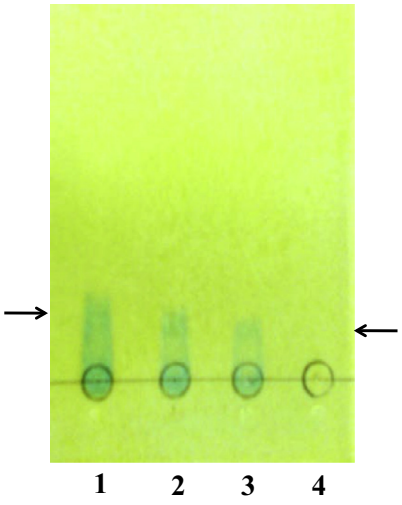

Fig. 2. Bioautographic thin layer chromatogram performed using culture supernatants of different *Streptomyces* spp. Twenty micro liters of supernatants of *S. coelicolor* (lane 1), *S. tendae* (lane 2), *S. aurantiacus* (lane 3) and *S. albaduncus* (lane 4) were spotted. Arrows point the blue spots used for assaying the lipase inhibition activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Lipase inhibition activities corresponding to the blue or greenish yellow regions of bioautographic TLC.

S. No.	Source of extract	Lipase inhibition (%)
1	Blue spot from the lane 3 of Fig. 1	76
2	Greenish yellow region from the lane 5 of Fig. 1	0
3	Blue spot from the lane 1 of Fig. 2	66
4	Blue spot from the lane 2 of Fig. 2	66
5	Blue spot from the lane 3 of Fig. 2	63
6	Greenish yellow region from the lane 4 of Fig. 2	0

value for the inhibitory spot of orlistat was 0.41. There was no blue zone in the lane (Fig. 1, lane 5) loaded with solvent (water) suggesting the specificity of the assay.

This assay was applied to screen the cultures of un-explored species of *Streptomyces* for the lipase inhibition activity. The details of the samples and the chromatogram development were described under Section 2. The cultures of *S. coelicolor, S. tendae* and *S. aurantiacus* have shown the lipase inhibitory blue zones against the greenish yellow back ground with the R_f values 0.2, 0.16 and 0.13, respectively (Fig. 2, lanes 1, 2 and 3). However, *S. albaduncus* culture did not show such enzyme inhibitory zone (Fig. 2, lane 4) suggesting the absence of the lipase inhibitors or their presence in traces. In order to confirm the presence of lipase inhibitor in the blue spots, silica gel at these spots was scrapped and extracted as described in Section 2. Extracts were used for assaying the lipase inhibition activity and it was present in the all the extracts from the blue zones, whereas no lipase inhibition activity was observed in the extracts obtained from the silica gel corresponding to the background and *S. albaduncus* (Table 1). These results suggest that this method will also be useful for the purification of the lipase inhibitor from the unknown sources.

Conflict of interest

Authors declare no conflict of interest.

Acknowledgments

Authors thank Dr. Chenraj Roychand, President, Jain University Trust and Dr. Krishna Venkatesh, Director, Centre for Emerging Technologies, Jain University for the research facilities.

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