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DEVELOPMENT OF A PAPER-BASED DEVICE USING INDOXYL ACETATE AS A CHROMOGENIC SUBSTRATE FOR RAPID QUALITATIVE DETERMINATION OF LIPASE INHIBITOR

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ABSTRACT

An improved paper-based device using indoxyl acetate as a chromogenic substrate was developed for rapid qualitative determination of lipase inhibitory activity. Colorless indoxyl acetate can be catalytically hydrolyzed by lipase to produce the blue-colored indigo dimer on the paper device. In the presence of a lipase inhibitor, enzyme activity diminishes, resulting in reduced hydrolysis of indoxyl acetate. Consequently, this leads to a decrease in the intensity of the blue coloration, which can be visually observed across a range of blue shades and analyzed using image analysis. Orlistat was used as the representative lipase inhibitor in the assay. The LODs obtained from visual detection and the image analysis were comparable. The paper-based device using indoxyl acetate for lipase inhibition assay was simple and convenient. It could be useful as a simple screening method for the detection of orlistat adulteration in weight loss supplements.

Keywords: paper-based device, lipase inhibitor, indoxyl acetate, orlistat

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Introduction

Pancreatic lipase (Lipase (EC 3.1.1.3), or triacylglycerol acyl hydrolases, belongs to the lipase esterase superfamily and plays a significant role in the digestion of triacylglycerol in the human body. The hydrolytic conversion of triglyceride-based lipids in fat-containing food to monoglycerides and free fatty acids by lipase facilitates the absorption of these substances into the body through the intestine.[1] The inhibition of this enzyme reduces the digestion of dietary fat, leading to decreased fat absorption into the body. Accordingly, recent approaches for the treatment of obesity focused on the inhibition of dietary triglyceride absorption via pancreatic lipase inhibition.[2] Despite extensive efforts to search for potential pancreatic lipase inhibitors, orlistat remains the only potent lipase inhibitor approved for weight control and obesity treatment.

The screening of natural resources for lipase inhibitors is therefore in need. The methods for detecting lipase inhibitors, including fluorescence assay[3], spectrophotometric assay[4,5], and TLC-based bioautographic method[6-9], have been reported. However, these methods require expensive equipment and laborious operations and often utilize organic solvents that are not environmentally friendly. Therefore, there is a need for a high sample throughput method for the screening of natural resources for lipase inhibitors. In our previous study, the colorimetric paper-based device for lipase inhibition assay, using naphthyl acetate as a substrate and fast blue B salt (FBB) as a chromogenic reagent, was developed for rapid and visual detection of the lipase inhibitor, orlistat, in weight loss supplements.[10] The method offers several advantages, such as simplicity, low cost, rapid analysis, and low consumption of reagents and samples. The method relies on lipase hydrolyzing naphthyl acetate to produce α-naphthol, which subsequently reacts with FBB to form a purple diazonium dye. When orlistat is present, the naked eye can visibly detect a reduction in the purple color on the paper-based device.

Recently, indoxyl acetate has been utilized as a substrate for a spectrophotometric assay of lipase activity.[11] Functioning as a chromogenic substrate, indoxyl acetate is initially colorless but can be enzymatically hydrolyzed by lipase to produce a blue-colored indigo dimer.[12] In this study, a paper-based device for the rapid detection of lipase inhibitory activity was developed using indoxyl acetate as the chromogenic substrate, with the aim of simplifying the process. Orlistat, a semisynthetic derivative of lipstatin, was employed as the representative lipase inhibitor in the assay. Renowned for its potent inhibition, orlistat permanently binds to the active site of pancreatic lipase, thereby hindering its hydrolytic function. Consequently, the presence of orlistat in the paper device is expected to reduce the hydrolytic breakdown of indoxyl acetate, leading to a decrease in the formation of indigo-blue color. The advantage of this method lies in its ability to visually observe and analyze the decrease in color intensity on the paper device without the need for additional visualizing reagents.

Materials and Methods

Chemicals and reagents

Lipase from porcine pancreas (L3126), orlistat (O4139), tris (hydroxymethyl) aminomethane (Tris, 154563), indoxyl acetate (I3500), calcium chloride and hydrochloric acid were all obtained from Sigma-Aldrich (St Louis, MO, USA). Four weight loss supplements were purchased through the Internet and from local stores. Analytical-grade reagents and deionized water were used throughout this experiment. Whatman No. 1 (W1) was purchased from GH Healthcare (Buckinghamshire, United Kingdom). Ethanol was obtained from Merck (Darmstadt, Germany).

Preparation of enzyme solutions

A concentrated stock lipase solution at 50 mg/mL in 50 mM Tris-HCl-$CaCl_2$ buffer pH7.5 was prepared according to the previously reported

method and kept at -20°C.[10,13] An aliquot amount of lipase stock solution was diluted with 50 mM Tris-HCl-$CaCl_2$ to the working solution at 5 and 10 mg/mL.

Preparation of indoxyl acetate solution and orlistat standard solution

A solution of indoxyl acetate at concentrations of 25 and 50 mM was prepared using ethanol as the solvent. For the orlistat standard solution, an appropriate amount of orlistat was dissolved in ethanol to achieve a 5 mg/mL stock solution. The stock solution was further diluted with ethanol to obtain a series of working orlistat solutions ranging from 0.0005-1.2 mg/mL and kept at 4°C until analysis.

Development and optimization of paper-based device

The paper with a flower-like pattern consisting of five circular detection zones and one circular zone at the center, each with a diameter of 7 mm, was designed. Each of the five zones was connected to the central zone through a 3 mm x 1.5 mm channel. The flower-like hydrophilic paper was simply fabricated by laser cutting and attached to a transparent hydrophobic plastic sheet using a 2x2 mm double-sided adhesive tape at the central zone. The experiments were carried out by adding 3 µL of indoxyl acetate into five detection zones, followed by being air-dried at room temperature for 5 min to remove ethanol. Then, 3 µL of lipase solution was subsequently added. The paper device was covered with a transparent plastic sheet to preserve humidity and left at room temperature (26 ± 2°C) for 20 minutes. Color intensity on the paper was captured by a digital scanner (Epson L3150). The scanned images were saved as a Joint Photographic Experts Group (JPEG) file at a resolution of 600 pixels and processed by using an open-source image processing program, ImageJ 1.54h software (National Institutes of Health, USA). The experiments, including lipase concentrations of 2.5, 5, and 10 mg/mL and indoxyl acetate concentrations of 25 and 50 mM, were performed. The conditions that showed good color intensity were further investigated for lipase inhibition assay by using orlistat as the representative lipase inhibitor. In the optimized study, 3 µL of 0.08 mg/mL orlistat was added at the detection zone. The relative percentage change between I_{ol} and I, where I_{ol} and I are the color intensity with and without orlistat, respectively, were calculated (rel. $\Delta I\% = (I_{ol}-I/I)*100$). Three replicates were performed for each experiment.

Performance of the paper-based device for lipase inhibition assay

The condition showing good color intensity was employed as follows. Three µL of 25 mM of indoxyl acetate was first pipetted into each of the five detection zones. The paper was left to dry at room temperature for 5 min. Then, 3 µL of orlistat solutions at concentrations of 0.0005-1.2 mg/mL was loaded into each circular zone of the paper, followed by being air-dried at room temperature for 5 min to remove ethanol. Then, 3 µL of 5 mg/mL lipase solution was subsequently added. The solvent must be removed entirely before adding the enzyme. If lipase is exposed to ethanol, it may lose its native structure and function. The paper device was covered with a plastic sheet and left at room temperature (26 ± 2°C). After 20 minutes, the paper device was scanned. The color intensity of the scanned image at each detection zone was measured. At each concentration, the color intensity data in terms of pixels were averaged from measurements of five detection zones. The limit of detection (LOD) was estimated by visual detection, and the LOD given by IC_{50}[14] was calculated from the semi-log curve between the color intensity and the concentrations fitted by the AAT Bioquest website program (https://www.aatbio.com/tools/ic50-calculator). Three sets of the paper device were performed.

The reproducibility of the device for orlistat detection at the LOD concentration was determined and reported as the relative standard deviation (%RSD) of the color intensity from six replicates. Each replicate was determined from five measurements at

the detection zone of each paper device. The lipase inhibitory activity of four weight loss supplements claimed as fat blockers was investigated. Each sample solution was prepared as reported previously.[10] Three µL of a clear sample was directly applied. Rel. ΔI% was calculated for each sample.

Data processing and interpretation

The ImageJ software was employed to process the scanned image by color thresholding, setting white as the threshold color and RGB as color space, followed by inverting it to 8-bit to measure the integrated density of the color in grayscale, which corresponds to the intensity of the blue color observed in the test zone on the paper device. In ImageJ, integrated density is a measurement that considers both the area and intensity of pixel values within a selected region of interest (ROI) in an image. Circular segment values of 180 x 180 width and height were applied to each detection zone. The mean integrated density of color, representing color intensity in terms of pixels within the selected area of an image, was determined based on five measurements of each test zone on the paper device.

Results and Discussion

To continually develop a simple screening method based on the paper device for lipase inhibition assay, indoxyl acetate was chosen as a substrate due to its cost-effectiveness and its capacity to serve as a self-chromogenic reagent. The colorimetric detection is based on lipase catalyzing the hydrolysis of indoxyl acetate, leading to the formation of an indigo-blue color (Figure 1). Therefore, it was expected that the intensity of indigo-blue coloration would decrease in the presence of a lipase inhibitor.

To explore this potential on the paper device, 25 and 50 mM indoxyl acetate substrate and lipase enzyme at 2.5, 5, and 10 mg/mL were examined to detect the blue color intensity at the test zone of the paper device. The results revealed that an indigo-blue color was visibly apparent when using indoxyl acetate at both concentrations of 25 and 50 mM. As the concentration of lipase increased, there was a noticeable enhancement in the intensity of the indigo-blue color, particularly strong at lipase concentrations of both 5 and 10 mg/mL (Figure 2a). Therefore, with the aim of minimizing reagent usage, the concentrations of indoxyl acetate at 25 mM and

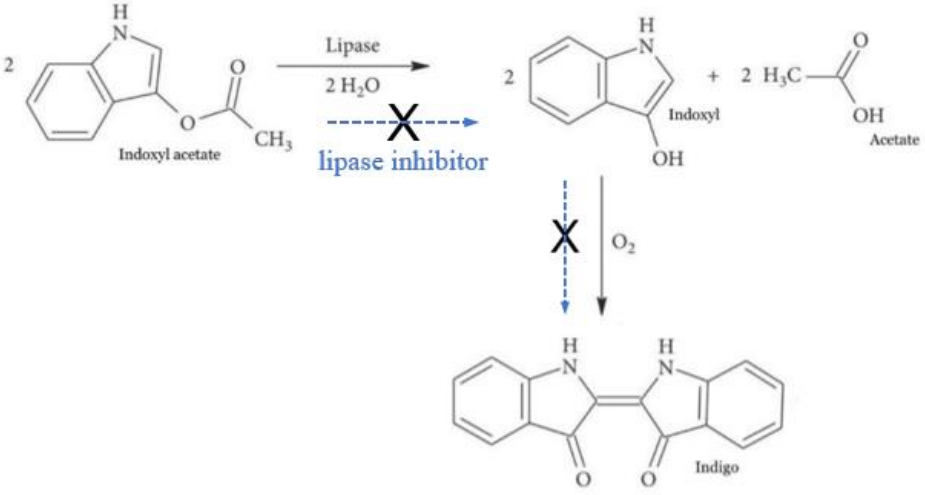

Figure 1 Reaction of lipase with indoxyl acetate

lipase at 5 and 10 mg/mL were selected for the subsequent investigation of lipase inhibitory activity, using orlistat as the representative lipase inhibitor in the assay. The findings showed that the presence of orlistat resulted in a noticeable decrease in the indigo-blue coloration on the paper device, observable to the naked eye (Figure 2b). Color intensity measurements were analyzed using ImageJ software to determine the reduction in color intensity, expressed as a relative percentage change (rel. ΔI%). The rel. ΔI% values (± S.D.) obtained from the conditions utilizing lipase concentrations of 5 and 10 mg/mL, fell within similar ranges of -50.3 ± 2.7 and -52.2 ± 6.2, respectively.

Therefore, the paper-based device, employing the condition of 5 mg/mL lipase and 25 mM indoxyl acetate, which exhibited good color intensity while minimizing reagent usage, underwent further evaluation against orlistat concentrations ranging from 0.0005 to 1.2 mg/mL. The findings showed that the color intensity of the device with orlistat at a lower concentration range (OL0.0005-OL0.002 mg/mL), measured by ImageJ, did not significantly decrease nor differ much compared to the one without orlistat. Consequently, the calculated rel. ΔI% for each replicate was relatively low, resulting in a large variation among three replicates. As the orlistat concentration increased (OL0.005-OL1.2 mg/mL), there was a noticeable reduction in the intensity of the indigo-blue color at the detection zone, which became more prominent and easily analyzed using ImageJ. However, the concentrations of orlistat ranging from 0.02 to 1.2 mg/mL displayed apparent saturation in the decrease of color intensity (Fig. 3a) which was similar to the findings in a prior study utilizing α-naphthyl acetate and fast blue B as a visualization reagent[10]. This suggests that the method may not be appropriate for quantitative purposes. Nevertheless, the device could be beneficial for qualitative analysis, as a noticeable decrease in blue coloration was clearly observed starting from a concentration of 0.005 mg/mL (Figure 3a).

To determine the detection limit of the device, the LOD by visual determination, as observed by an apparent reduction in color intensity, was approximately 0.005 mg/mL (Fig. 3a). This visual observation provided comparable performance to the LOD calculated from the semi-log plot between the color intensity versus various orlistat concentrations (Fig. 3b). The calculated LOD was defined as the IC_{50}[14], obtained from a semi-log plot fitting by AAT Bioquest. AAT Bioquest employs a four-parameter logistic curve fitting method to fit a sigmoidal curve to the data points. The IC_{50} value was

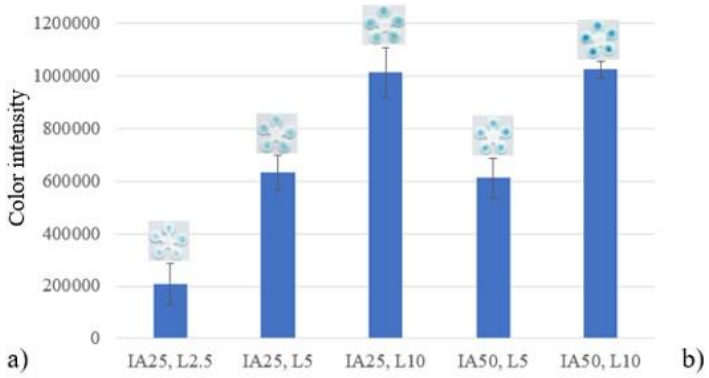

Figure 2 a) Color intensity at the test zone of the paper device under various concentrations of indoxyl acetate (IA) and lipase (L). b) Paper-based analysis and rel. ΔI% of color intensity without and with 0.08 mg/mL orlistat. Data are the means (± SD) of three replicates.

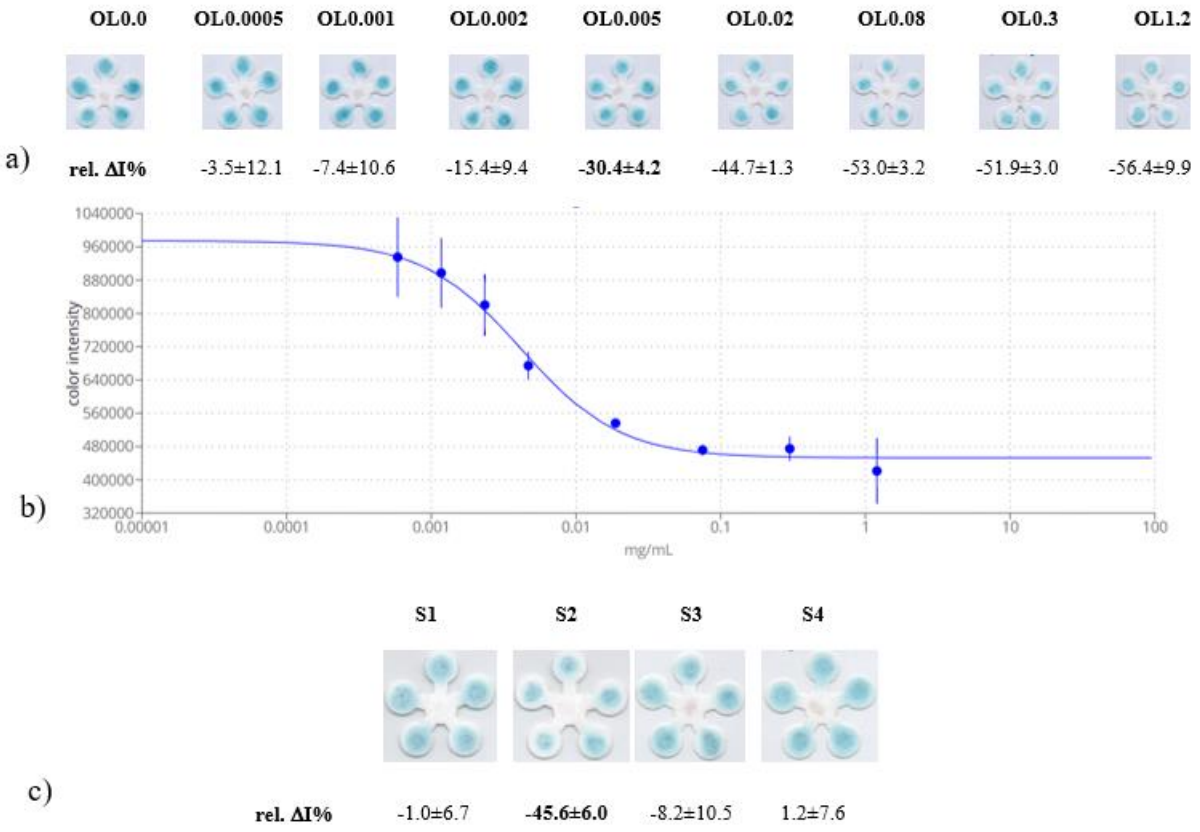

Figure 3 a) Paper-based analysis and rel.ΔI% of different orlistat (OL) concentrations. b) The semi-log curve between the color intensity and orlistat concentrations fitted by the AAT Bioquest. c) Paper-based analysis of weight loss supplements, S1-S4. Data are the means (± SD) of three replicates.

calculated from the curve as the concentration at which the response is halfway between the maximum and minimum response observed.[15] The calculated LOD, given by IC_{50}, was found to be 0.005 ± 0.001 mg/mL. A reproducibility of the device for orlistat detection at the concentrations of 0.005 mg/mL was achieved with a %RSD of 6.6.

The paper device was further employed to evaluate the lipase inhibitory activity of four weight loss supplements (S1-S4), which had previously been analyzed using α-naphthyl acetate and Fast Blue B for colorimetric detection in the paper-based assay.[10] Both methods produced similar results, with only S2 showing a noticeable reduction in color intensity (Figure 3c), confirming its adulteration with orlistat as reported earlier. Interference from other substances in the samples did not inhibit the enzymatic reaction on the paper device to the same extent as observed with orlistat. As a result, the decrease in the indigo-blue color observed in S1, S3, and S4 was much less visible, indicating the absence of any potential lipase inhibitor in these samples.

Conclusion

A paper-based device for rapid detection of lipase inhibitors utilizing indoxyl acetate was developed. When tested with a strong lipase inhibitor such as orlistat, the paper device demonstrated a decrease in indigo-blue color intensity, which was easily detectable through visual inspection or image analysis software. Both visual detection and image analysis yielded comparable limits of detection (LODs). Compared to the previous method utilizing α-naphthyl acetate and Fast Blue B as a visualization reagent[10], the proposed method demonstrated similar results while simplifying the process by

eliminating the need for an additional visualization reagent. Furthermore, the device was easily constructed from filter paper using low-cost materials with minimal resource consumption and involved straightforward procedures. The method could be useful for qualitatively detecting orlistat adulteration and screening lipase inhibitory activity from various sources. However, further investigation into the stability testing of the enzyme and chromogenic substrate in their dried forms on the paper should be considered, as it could prove beneficial in preparing this analytical platform for on-site analysis.

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