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Full length Article

A simple method to screen amylase inhibitors using thin layer chromatography

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ABSTRACT

A less-time consuming and more specific qualitative technique to screen for α-amylase inhibitors could facilitate high throughput screening. Taking up developing method facilitating this as an objective, a protocol to screen a large number of plant extracts for presence of α-amylase inhibitors is being reported here. The potential of separated compounds to inhibit α-amylase was checked by first flooding their TLC plate with a solution of α-amylase followed by a solution of the substrate, starch. Iodine staining was then used to visualise inhibitory activity where a blue colour spot on the TLC plate was taken as a positive α-amylase inhibitory test for the corresponding compound. The visualization was enhanced by observing the plate under UV light. Blue spots were observed on the TLC plate following visualization under UV light indicating presence of α-amylase inhibitory activity in the corresponding compound as a result of inability of α-amylase to hydrolyse starch. This inability we conclude was a result of inhibition of the enzyme by the compound on the TLC plate. Our protocol presents itself as a simple, less time consuming and more specific alternative for high throughput screening of various plants extracts without the use of any sophisticated instrumentation facility.

Keywords: α-amylase, α-amylase inhibitors, Diabetes, *Ocimum sanctum*, Thin Layer Chromatography (TLC).

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder which results due to chronic hyperglycemia associated with the imbalance in carbohydrate, fat and protein metabolism (Lebovitz, 1997), with a significant impact on the health, quality of life and expectancy of patients (Know *et al.,* 2008). International Diabetes Federation (IDF) estimates that as of 2013, 382 million people worldwide are diabetic with the number expected to rise to 592 million by 2053 (Murphy, 2013). One of the therapeutic approaches for treating diabetes is to decrease postprandial hyperglycemia which is mediated by two enzymes, α-amylase and α-glucosidase. Inhibition of these enzymes can reduce postprandial hyperglycemia through delay of enzymatic hydrolysis of carbohydrates, thus reducing absorption of glucose in the digestive tract (Yadahally *et al.,* 2012). Existing powerful synthetic inhibitors like acarbose, voglibose etc., can cause hepatic and gastrointestinal disorders (Murai *et al.,* 2002). This warrants search for alternative compounds that do not cause such side effects. Plants have always been an exemplary source of drugs and the ethno botanical information reports about 800 plants that may possess antidiabetic potential (Alarcon-Aguilar, 1998). So, screening of plant extracts for novel compounds that can inhibit amylases arouses interest and points towards a promising future. There are mainly two types of assays which are most commonly used to determine the activity of α-amylase and glucoamylase. One is based on measuring the amount of reducing sugars by the dinitrosalicylic acid (DNS) assay (Miller, 1959; Zhizhuang *et al.*, 2006) or the Nelson–Somogyi method (Najafi et al., 2005; Primarini *et al.,* 2000),

where as the other is based on the decreased staining value of blue starch–iodine complexes (Fuwa 1954; Manonmani *et al.,* 1999). The analytical methods currently available to determine the activity of α-amylase have several disadvantages. The methods do not have satisfactory specificity like compounds in the sample which is responsible for the inhibition. Multiple steps and time consumption are other disadvantages. The research reported herein is an attempt to overcome these disadvantages by developing a simple technique that could facilitate a high throughput screening up to the level of compound specificity. Our method, thus, presents itself as a powerful alternative technique that allows high throughput screening of plant samples for presence of α-amylase inhibitory activity to the extent of compound specificity. The plant samples used are extracts of *Ocimum sanctum* which has been chosen because of its known antidiabetic medicinal plant records in Ayurveda (Palla *et al.,* 2012).

MATERIALS AND METHODS

Chemicals

Starch, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, *α*-amylase, and Gram's Iodine was purchased from Hi-Media Laboratories, Mumbai, India. Solvents like petroleum ether, ethyl acetate, chloroform, acetone, ethanol, toluene, and methanol were from Qualigens. TLC Silica gel 60 F254 plates were purchased from Merck.

Plant material

Dried powder of *Ocimum sanctum* plant was purchased from the local market of Nanded city, India which was used for the further study.

Preparation of Plant extracts

20 gm of sample was successively extracted in an order of non-polar to polar solvent based on increasing degree of polarity. The different extracts obtained sequentially were with petroleum ether, ethyl acetate, chloroform, acetone, ethanol and distilled water, respectively. Extractions were performed using Soxhlet apparatus. The temperature maintained in each extraction was 10-20 0C lower than the melting point of the solvents used. The extracts obtained were concentrated by boiling and were stored in a refrigerator until further use.

Inhibition assay

Thin-layer chromatography was performed on the TLC Silica gel 60 F254 plates (Merck KGaA, Germany). The extracts were spotted on the plates using a micropipette and allowed to dry. One-dimensional TLC analysis was performed with toluene: chloroform: methanol in volume ratio 45: 50: 5 respectively as mobile phase. Spots were observed under Ultra-Violet light (UV light) at 254 nm and 366 nm. The TLC plates were then incubated in the amylase solution for 30 min for primary reaction between the enzyme and inhibitor. After incubation, the plates were taken out of the amylase solution and incubated in 1 % starch buffer of pH 6.9 for 10-20 min for enzyme-substrate reaction. The plates were then washed with Gram's Iodine solution and observed. These experiments performed in triplicate to check the reproducibility of the method.

RESULTS AND DISCUSSION

Six different sequential extract of sample plant were obtained using the solvents petroleum ether, ethyl acetate, chloroform, acetone, ethanol and water respectively which revealed different bands when TLC was performed. Out of the mentioned extracts screened by our method, chloroform and ethanol extracts contained inhibitors (figure 1 and figure 2). The plant extracts, positive for α-amylase inhibitors, showed blue stains upon iodine staining at the positions of separated bands on the TLC plate, indicating the separated fraction (band) was responsible for the inhibitory activity in the corresponding extract. This reaction we attribute to the starch-iodine complex formation resulting from starch which was not hydrolysed by the enzyme due to its inhibition by the compound and hence got stained blue by iodine on that position. The TLC analysis revealed nine bands in chloroform extract and ten bands in ethanol extract when observed under UV light. After performing out test as described above, blue spots were observed on three positions on the TLC plates of chloroform extracts and six positions on that of ethanol. As observed in chloroform extract TLC plate (figure 1), blue spot was observed on the position of the fourth, fifth and ninth band with the fourth band position showing darker colour as compared to other two. In ethanol extracts (figure 2), the first, fourth, sixth, seventh, eighth and ninth band on TLC plate were the positions showing blue colour

Fig. 1: Chloroform extract contained amylase inhibitors.

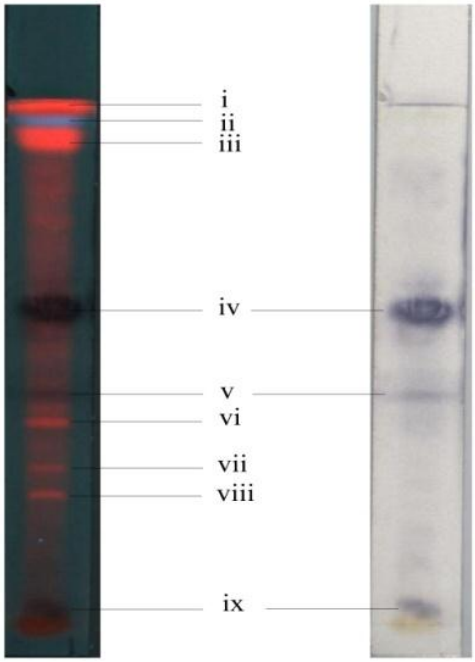
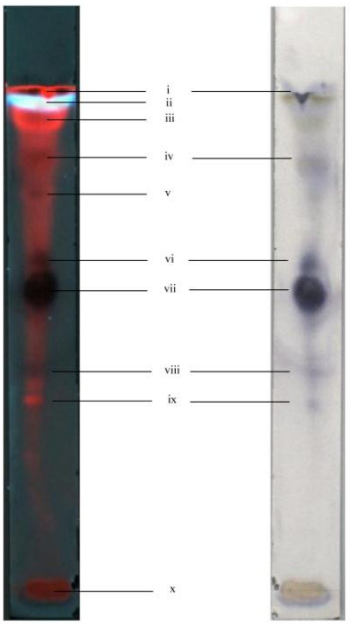


Fig. 2: Ethanol extract contained amylase inhibitors.



with the sixth band showing darker colour on its position compared to other five bands. So, the technique we report herein exploits the traditional starch-iodine stain method to visualize the hydrolysis of starch by an enzyme pre-adsorbed onto a TLC plate of separated compounds from an extract, which could or could not inhibit the enzyme. Our method is simple and can be used as a rapid detection method for presence of α-amylase inhibitors, based on starch–iodine complex reaction. Another advantage of our method is that it does not require any instrumentation facility. This coupled to the less time consumption i.e. roughly an hour from TLC performance onwards, makes our technique, highly suitable for high throughput screening of plant samples for presence of enzyme inhibitors. However, the real merit of this procedure lies in its specificity to the level of compound/s in the extract responsible for inhibitory activity.

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