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Reverse Phase Compatible TLC-Bioautography for Detection of Tyrosinase Inhibitors

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ABSTRACT:

Introduction – Reverse phase chromatography and bioautographic assays are key tools for natural product bioguided isolation; however, their direct coupling has not been fully achieved.

Objectives – To develop a bioautographic assay to detect tyrosinase inhibitors present in complex matrices sorbed on reverse phase (RP) TLC-plates that can be used for bioguided isolation of bioactive compounds.

Methods – Enzyme gel entrapment with an amphiphilic copolymer was used for assay development. The gel turns into a brown "skin like" colour due to tyrosinase catalysed oxidation of L-tyrosine. The inhibitors are visualised as clear spots against a brown coloured background.

Results – The assay was able to localise cinnamaldehyde in *Cinnamomum cassia* essential oil, as its main constituent with known tyrosinase inhibition properties. The assay allowed the detection of 0.03% (*w*/*w*) of kojic acid co-spotted with a methanolic extract of *Sphaeralcea bonariensis* and chromatographed on RP-TLC.

Conclusion – The developed assay is able to detect, with high sensitivity, tyrosinase inhibitors present in complex matrices that were chromatographed in RP-TLC. Results can be easily read by colour change, inhibitors appear as clear spots in a darker background.

Keywords: TLC-bioautography; herbal drugs; tyrosinase inhibition; dermatological disorders; poloxamers

Introduction

Tyrosinase (EC 1.14.18.1) is a metalloprotein belonging to the type 3 copper-containing protein family (Decker and Tuczek, 2000), which is widely distributed throughout the phylogenetic scale, from bacteria to humans (Selinheimo *et al.*, 2007). The enzyme has a bifunctional catalytic mechanism: (i) the ortho-hydroxylation of monophenols to ortho-diphenols (monophenolase activity) and (ii) the oxidation of ortho-diphenols to ortho-quinones (diphenolase activity). The resulting reactive ortho-quinones polymerise to melanin (Sánchez-Ferrer *et al.*, 1995). In humans, melanin is responsible for the colouring of skin and hair, and helps to protect the skin from the damage caused by ultraviolet radiation (Brenner and Hearing, 2008). However, excessively high levels of melanin can cause various dermatological disorders such as melasma, age spots and sites of actinic damage (Kim and Uyama, 2005). Other diseases, including cancer and Parkinson's disease, are also characterised by abnormalities in tyrosinase activity (Pan *et al.*, 2011). Additionally, tyrosinase is responsible for enzymatic browning of fruits and vegetables during senescence or post-harvest handling (Martinez and Whitaker, 1995). In insects tyrosinase is involved in wound healing, and parasite encapsulation and sclerotisation, playing an important role in developmental and defensive functions (Andersen, 2010).

Consequently, tyrosinase inhibitors have potential applications for improving quality of foods, insect pest control and prevention and treatment of melanin-related health problems in humans.

Natural resources have been an important supply of tyrosinase inhibitors. The resulting compounds work either directly as inhibitors or serve as model substances for the development of synthetic inhibitors (Loizzo *et al.*, 2012; Chen *et al.*, 2015; Lee *et al.*, 2016). Screening of natural product sources is a difficult effort with a high probability of isolation of known bioactive compounds. The initial extract of the natural material usually consists of a complex mixture. It may contain only very small quantities of a bioactive substance, often as a mixture with structurally related molecules (Li and Vederas, 2009).

TLC-bioautographic assays, are especially apt for detection of bioactive compounds present in complex mixtures, and for connecting bioactivity to chromatographic behaviour (Cieśla *et al.*, 2015). Such properties can be useful to guide fractionation of natural mixtures during the search for novel lead compounds (Cieśla *et al.*, 2015; Dewanjee *et al.*, 2015; Choma and Grzelak, 2011).

There are a few bioautographic assays available for the screening of tyrosinase inhibitors. Most of them are direct methods wherein an enzyme suspension is applied onto the TLC by spraying or dipping (Wangthong *et al.*, 2007; Taibon *et al.*, 2015). One common limitation of these assays is their lack of compatibility with reverse phase (RP) TLC. Despite being a key tool for the isolation of small molecules from complex mixtures, RP chromatography is hardly found coupled to bioautographic assays. The hydrophobicity of RP-TLC plate surfaces hampers the deposition of a homogeneous aqueous layer containing the enzyme and the reagents required for the assay. Sometimes, the poor

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wettability of lipophilic compounds adsorbed on normal phase TLC surfaces can produce false results in bioautographic assays (Taibon *et al.*, 2015).

Recently, we achieved enzyme immobilisation by agar gel entrapment to set up a tyrosinase TLC-bioautography (García and Furlan, 2015). Although an homogeneous agar gel layer is easily formed on a normal phase TLC surface, its formation is more difficult on a RP-TLC surface. In addition, the repulsion between the hydrophilic and the hydrophobic layers at the interface between the gel and the surface hampers the migration of compounds from the TLC to the gel. This problem has been solved for an acetylcholinesterase TLC assay using gels with amphiphilic properties (Ramallo *et al.*, 2015a).

Because of the importance of RP chromatography for natural products purification, and the utility of TLC tyrosinase-bioautography to guide the isolation of natural inhibitors, in this paper we report a bioautographic assay for the detection of tyrosinase inhibitors employing poloxamer to immobilise tyrosinase on RP-TLC plates.

Experimental

Materials

Mushroom tyrosinase was purchased from Sigma-Aldrich (St Louis, MO, USA), kojic acid was purchased from MAGEL SA (Buenos Aires, Argentina).

Pluronic® F-127 (poloxamer 407) was donated by BASF Argentina S.A. (Buenos Aires, Argentina). Aluminium-backed silica gel 60 RP-18 F_{254} TLC layers were purchased from Merck (Darmstadt, Germany). *Cinnamomum cassia* (Nees & T. Nees) J. Presl (Lauraceae) essential oil was purchased from EUMA (Buenos Aires, Argentina).

Software

GelPro 3.0 (Media Cybernetics, Silver Spring, MD, USA) was used for measuring signals for inhibition spots intensity. VisionCATS release number 1.4.0 (Muttenz, Switzerland) was used to operate CAMAG equipment.

Sphaeralcea bonariensis (Cav.) Griseb. (Malvaceae) methanol extract preparation

The plant material was collected in Pergamino, Buenos Aires. A voucher specimen was deposited at the Herbarium of the National University of Rosario (ID MO018). The plant material was oven dried (at 65 °C for 72 h) and milled. The powdered plant material was extracted in refluxing methanol (5% (*w/v*)) for 45 min, three times. The filtered extracts were combined and evaporated to dryness under reduced pressure by rotary evaporation to yield 38 g (15%) of a brownish sticky *Sphaeralcea bonariensis* extract (SB extract).

Solutions

Phosphate buffer (20 mM) was prepared in distilled water adjusted to pH 6.8. Tyrosinase stock solution (1715 U/mL) was prepared suspending 11 mg of lyophilised enzyme (3130 U/mg) in 20 mL of 20 mM phosphate buffer pH 6.8. Pluronic® 20% (*w/w*) was prepared in phosphate buffer (pH 6.8) and kept at 4 °C until used.

Kojic acid (0.05 mg/mL) stock solution was prepared in ethanol. Five serial two-fold dilutions were freshly prepared by dilution of the kojic acid stock solution. *Sphaeralcea bonariensis* stock solution was prepared by redissolving 10 mg SB extract in 1 mL methanol. A stock solution of the essential oil of *C. cassia* was prepared in a concentration of 50 mg/mL in methanol.

TLC development

The chromatograms were acquired and processed using equipment from CAMAG (Muttenz, Switzerland). In the first step an image of the clean RP-18 W TLC plate was captured under white light and UV_{254nm} and UV_{365nm} with the CAMAG TLC Visualiser. 10 μL of each sample were applied as a band (5 mm) onto the TLC plate using the CAMAG Automatic TLC Sampler 4 (ATS 4) under nitrogen flux. The TLC plate was developed using the Automatic Developing Chamber 2 (ADC 2), mobile phase depended on the case. The image of the TLC was captured again under white light and UV_{254nm} and UV_{365nm} with the CAMAG TLC Visualiser.

TLC analysis of the *S. bonariensis* extract was carried out by spotting 10 μL of a 10 mg/mL extract solution on the RP-TLC plate. In co-spotting experiments 10 μL of the appropriate solution of kojic acid were spotted. Mobile phase: methanol: water (1:1).

TLC analysis of the *C. cassia* essential oil was carried out by spotting decreasing volumes of 50 mg/mL solution, in the range 600 μg to 100 μg. Mobile phase: methanol/water (1:1).

Typical assay procedure

At 4 °C, 452 μL of tyrosinase solution 1715 U/mL and 1.8 mg of tyrosine were added to 16 mL of Pluronic® (20% (*w/w*), pH 6.8) and mixed carefully by flipping. This staining solution was immediately poured directly on a RP-TLC plate (100 cm^2), previously developed in the corresponding mobile phase, and allowed to reach room temperature to produce a colourless gel. Within 2 min, the gel turns into a brown "skin like" colour due to the oxidation of L-tyrosine by tyrosinase.

Detection of decreasing amounts of kojic acid

Amounts of Kojic acid (1000, 500, 250, 125, 62.5, 31.25, and 15.63 ng) were spotted, onto 3 × 11 cm RP-TLC plates. Bioautography was carried out without prior chromatographic separation.

Results and discussion

Achieving an homogeneous dispersion of enzyme suspension layers on hydrophobic surfaces is difficult because of the poor wettability of such surfaces. This situation can create problems in enzyme bioautographic assays. If the aqueous enzyme solution is not distributed in a uniform way over the whole TLC surface, inhomogeneities of the background result, leading to differences in background colouration and formation of unequal colours. This can produce anomalous results for lipophilic compounds adsorbed on normal phase TLC plates. Although in normal phase TLC such problems have been avoided with the addition of triton (Taibon *et al.*, 2015), this difficulty has hampered the coupling of bioautographic assays to RP-TLC.

An alternative way of depositing an enzyme on TLC plates for bioautographic assays is to generate a thin gel layer containing enzyme and reagents (immobilisation by gel entrapment). This strategy has been applied for the development of xanthine oxidase (Ramallo *et al.*, 2006), β-glucosidase (Salazar and Furlan, 2007), tyrosinase (García and Furlan, 2015) and acetylcholinesterase TLC-assays (Ramallo *et al.*, 2015a, 2015b). Depending on the conditions, this strategy can increase enzyme stability and improve reproducibility, facilitating uniform enzyme coverage of the TLC surface by knowing the exact final concentration of enzyme that is applied per unit area.

Although agar gels have been more broadly used for this purpose, gels with amphiphilic properties such us poloxamer gels may be a better choice when working with RP-TLC plates.

Poloxamers are non-ionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). They can form temperature dependent micelles that facilitate solubilisation of hydrophobic compounds. Water solutions of poloxamer show thermo-reversible gelation behaviour (Bohorquez *et al.*, 1999; Higuchi *et al.*, 2005): they are sols below room temperature and become gels at 37 °C (Ruel-Gariépy and Leroux, 2004). In addition, poloxamer gels can help to preserve enzymes from alkaline stress and aggregation, and to increase their thermal stability (Pucciarelli *et al.*, 2012). The use of poloxamer for a RP-TLC bioautography has been recently employed for the first time in an acetylcholinesterase assay (Ramallo *et al.*, 2015a).

Assay development

In a recently published tyrosinase bioautography (García and Furlan, 2015), the variables incubation time, incubation temperature, pH, as well as the amount of substrate and enzyme were optimised for normal phase TLC employing agar as gelling agent. These conditions were initially kept using poloxamer gel instead of agar gel, and RP-TLC as stationary phase instead of silica gel. The enzyme was suspended in buffered solution (pH 6.8) containing substrate and poloxamer. After careful mixing, the suspension was distributed on a RP-TLC layer at 4 °C, and the plate was warmed up to 37 °C on a hot plate. After gel formation, the plate was incubated at 37 °C during 20 min in a stove. As the enzymatic reaction proceeded, dyeing developed, and the initially colourless gel changed to brown because of the generation of ortho-quinones (Fig. 1) that polymerised to generate brown coloured products. Inhibitors sorbed on the TLC plate were visualised as white spots under visible light.

Detection of kojic acid

In order to determine the sensitivity of the assay, decreasing amounts of the known inhibitor kojic acid were spotted on the RP-TLC under nitrogen flux. Enzyme inhibition provoked by the positive control was detectable down to 31.3 ng (Fig. 2), making this test as sensitive as the reported method for normal phase employing agar (García and Furlan, 2015).

Applicability to complex mixtures

The applicability of the assay to analyse bioactive natural active complex mixtures was studied employing *C. cassia* essential oil, reported as tyrosinase activity inhibitor (Chang *et al.*, 2013). *Cinnamomum cassia* essential oil was chromatographed and the poloxamer solution containing tyrosinase and L-tyrosine, was distributed over the plate. The bioautography showed the presence of one inhibition spot with Rf = 0.77 in acetonitrile/water (8:2) corresponding to cinnamaldehyde (Fig. 3).

Natural extracts are complex matrices wherein actives compounds can be present at different concentrations. In some cases, the active compound is present in relatively high concentration, whereas sometimes, the active compound is a minor constituent. In order to test the applicability of the assay for the localisation of an active compound in the presence of different proportions of a complex matrix, different amounts of kojic acid were co-spotted with an inactive crude extract of *S. bonariensis*. The SB extract was used as matrix because it showed, by TLC analysis, the presence of a variety of components, including spots with similar chromatographic behaviour to kojic acid. Samples were analysed by RP-TLC in parallel and the plate was stained using the tyrosinase inhibition assay.

The kojic acid and the extracts were chromatographed using methanol/water (1:1) mobile phase, and analysed under UV_{365nm} and UV_{254nm} before bioautographic assay (Fig. 4). When the bioautography was applied, an inhibition spot was clearly observed at Rf = 0.51 corresponding to kojic acid even for the lane that contained 0.03% (*w/w*) of the inhibitor (Fig. 4(a), lane 6); none of the components of the *S. bonariensis* extract interfered with the colour reaction. These results showed that the assay is compatible with RP-TLC allowing the sorption of the compounds on alkyl chains of a RP. The positive control kojic acid was detected on the TLC plate down to 31.3 ng, which makes the test comparable to the reported method for silica gel plates coupled to tyrosinase activity detection in agar gel (García and Furlan, 2015).

The RP-TLC tyrosinase bioautography described herein is rapid and simple to use. It shows good background-spot contrast and very good sensitivity,

One-dimensional TLC experiments allow qualitative activity analysis of several plant extracts at the same time, being especially useful for the identification of bioactive compounds present in other complex mixtures such as chemically engineered extracts (Lopez *et al.*, 2007; Ramallo *et al.*, 2011).

Finally, poloxamers could be applicable to other bioautographic assays in RP-TLC. Its thermal reversibility allows enzyme solution storage, and the temperature change required for gel formation is around 10 °C: poloxamer 407 water solutions are liquid below 4 °C and form a gel at 14 °C. Such characteristics make these block copolymers particularly suited to develop assays with enzymes that are sensible to temperature.

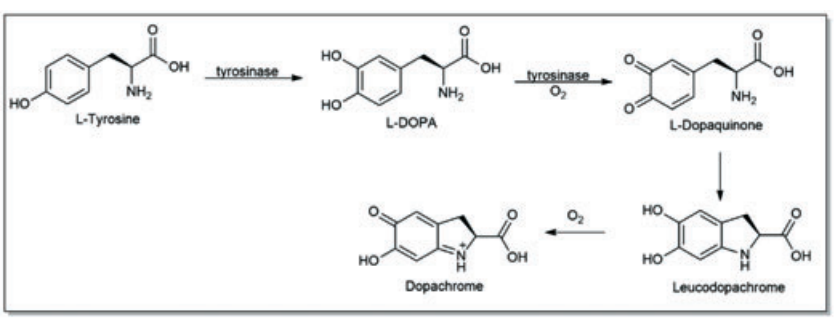

Figure 1. Tyrosinase catalysed hydroxylation and oxidation reactions.

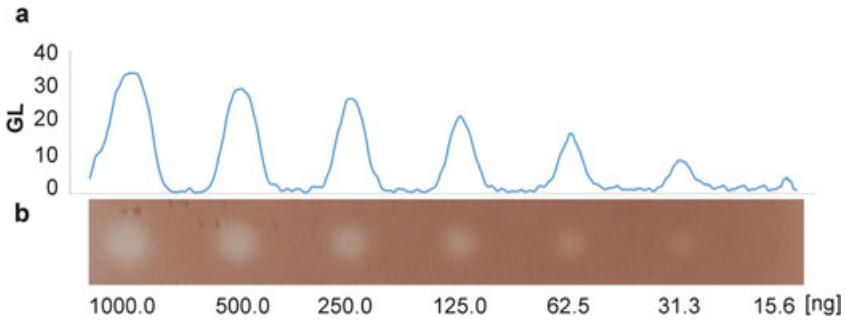

Figure 2. Tyrosinase activity inhibition detected on a TLC layer spotted with decreasing amounts of kojic acid and stained with the bioautographic assay. (a) Optical density of the spots measured by Gel-Pro software. (b) TLC-bioautography observed under visible light.

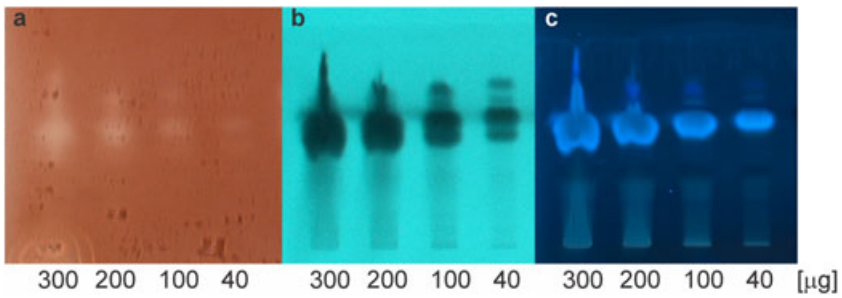

Figure 3. Bioautography for detection of tyrosinase inhibitors on RP-TLC for *C. cassia* essential oil. (a) Tyrosinase bioautography visualised under visible light; (b) UV_{254nm} light detection; (c) UV_{365nm} light detection. Elution was carried out with acetonitrile/water (8:2).

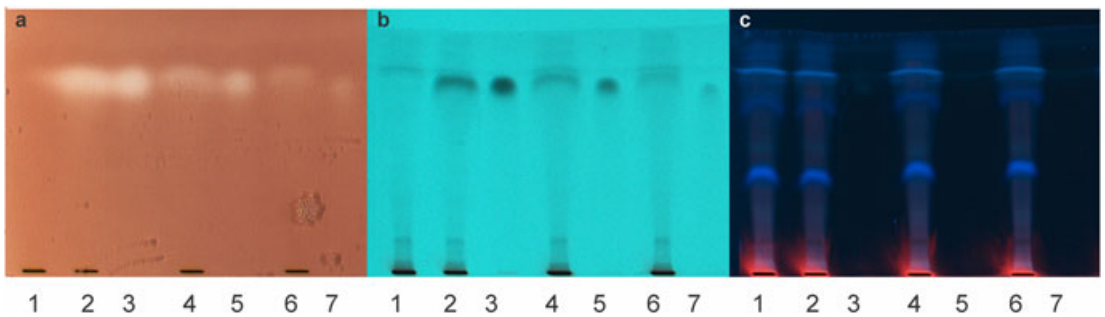

Figure 4. RP-TLC analysis of tyrosinase inhibitor kojic acid and *S. bonariensis* extract.From left to right, SB extract (100 mg) (1), co-spotted SB extract (100 µg) and kojic acid (0.125 µg) (2), kojic acid 0.125 µg (3), co-spotted SB extract (100 µg) and kojic acid (0.0625 µg) (4), kojic acid 0.0625 µg (5), co-spotted SB extract (100 µg) and kojic acid (0.03125 µg) (6), kojic acid 0.03125 µg (7). (a) Tyrosinase bioautography visualised under visible light; (b) UV_{254nm} light detection; (c) UV_{365nm} light detection. Elution was carried out with methanol/water (1:1).

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