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Detection of estrogen active compounds in hops by planar yeast estrogen screen

Dinah Schick, Wolfgang Schwack*

Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, D-70599 Stuttgart, Germany

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ABSTRACT

Hops used in the brewing process of beer for flavoring are known to contain estrogen active compounds (EAC) and to be the source of EAC in beer. The recently developed planar yeast estrogen screen (pYES) with the substrate resorufin-β-D-galactopyranoside (RGP) successfully was applied for the detection of EAC in ethanolic extracts of hops pellet samples. The only pYES positive compound was identified as the hop flavanone prenylnaringenin (PN) by thin-layer chromatography–mass spectrometry. The heat-induced formation of estrogen active PN from the inactive hop flavonoid desmethylxanthohumol was confirmed by simulation of wort boiling, extraction of both the hops' remainder and the supernatant water, and subsequent investigation of the extracts by pYES. By means of the dose-response curve of PN of a hops' remainder extract, the estradiol equivalent concentration (EEQ) and thus the estradiol equivalent amount (EEA) of PN in the hops' remainder after simulation of the wort boiling was determined to 39 μg/L and 52 μg/kg, respectively.

1. Introduction

The presence of estrogen active compounds (EAC) in hops is known for years and impacts on humans were observed as, for example, female hops pickers suffered from menstrual disturbances [1]. Milligan et al. identified four hop flavonoids as EAC, the prenylflavanones 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN) and 6,8-diprenylnaringenin, and the geranylated flavanone 8-geranylnaringenin, when 8-PN was identified as the most potent phytoestrogen [2,3]. The major hop flavonoid is xanthohumol (XN), while other flavonoids only occur in trace amounts [4], but the formation of isoxanthohumol (IXN) from XN and the estrogen active compounds 6-PN and 8-PN from desmethylxanthohumol (DMX) – inter alia during the brewing process of beer – was reported [5,6]. Thus, more prenylflavanones (partly with estrogenic activity) than prenylchalcones occur in beer [7].

In the present work, hops pellet samples were extracted with ethanol, and the extracts were investigated by planar yeast estrogen screen (pYES) to detect EAC [8]. Additionally, a part of the brewing process was simulated to verify the possible formation of EAC during heat supply in aqueous solutions. Both the hops' remainder and the aqueous phase were extracted, analyzed by pYES, and the results compared to them obtained with the original hops samples. The applied pYES provided the separation of the samples by high-performance thin-layer chromatography (HPTLC) prior to the bio-detection by yeast cells of a recombinant yeast strain of *S. cerevisiae* generated by McDonnell et al. [9,10], containing the human estrogen receptor and a reporter gene that encodes for β-D-galactosidase. By cleavage of the suitable substrate resorufin-β-D-galactopyranoside (RGP) by the enzyme that is produced in presence of EAC, orange fluorescent resorufin is released in the HPTLC zone allowing the clear detection of an estrogenic activity of a substance [8]. Moreover, detected EAC were identified by HPTLC coupled to electrospray ionization mass spectrometry.

In a recent work, the applicability of the logit-log method as evaluation tool for pYES was demonstrated, allowing the easy determination of half maximal effect doses (ED_{50}) of EAC and the calculation of estradiol equivalent factors (EEF), using the example of 17α-ethinylestradiol (EE2), a synthetic estrogen used in contraceptives [11]. Additionally, the use of pYES for the estimation of estradiol equivalent concentrations (EEQ) of unknown EAC in liquid samples or sample extracts with the EC_{50} method was taken into account [11]. These calculations were used in the present study to exemplarily estimate the EEQ of hops' remainder extracts and hence the estradiol equivalent amount (EEA) of the hops' remainder.

* Corresponding author.
E-mail address: wolfgang.schwack@uni-hohenheim.de (W. Schwack).

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2. Materials and methods

2.1. Chemicals and materials

The following chemicals and materials were from Merck (Darmstadt, Germany): potassium chloride (>99%), potassium dihydrogen phosphate (p.a.), copper(II) sulfate pentahydrate (p.a.), sodium hydroxide solution (20%), disodium hydrogen phosphate (p.a.), L-histidine (≥99%), hydrochloric acid (37%), and HPTLC glass plates silica gel 60 RP-18 W (200 mm × 100 mm, layer thickness 200 μm). Yeast nitrogen base without amino acids (YNB), casamino acids [12], adenine and agar for plate agar were from Becton Dickinson (Heidelberg, Germany). YNB without amino acids for liquid culture media, ethanol (≥99.8%), and methanol (≥99.9%) were obtained from Sigma-Aldrich (Steinheim, Germany). D-Glucose (anhydrous) and sodium hydrogen carbonate were purchased from BDH Prolabo Chemicals (VWR, Bruchsal, Germany). Ammonium formate (≥99%), acetonitrile (≥99.9%), diethyl ether (≥99.8%, ~2% ethanol as stabilizer), L-lysine (≥98%), and magnesium sulfate heptahydrate (puriss. p.a.) were obtained from Fluka-Sigma-Aldrich (Steinheim, Germany). Resorufin-β-D-galactopyranoside (RGP) was from Santa Cruz biotechnology (Dallas, USA), formic acid (98%) and dimethyl sulfoxide (DMSO, 99.98%) from Fisher Scientific (Schwerte, Germany). Acetone (≥99.8%) was from Carl Roth (Karlsruhe, Germany). Ultrapure water was supplied by a Synergy System (Millipore, Schwalbach, Germany). Yeasts of the strain *Saccharomyces cerevisiae* BJ3505 (protease deficient, MATα, PEP4::HIS3, prb1-Δ1.6R, HIS3-Δ200, lys2-801, trp1-Δ101, ura3-52gal2can1) for pYES were generated by McDonnell et al. [9,10] and stored in a cryo-vial at −70 °C. Orange filter glass O 580 from HEBO (Aalen, Germany) was used as optical filter for the TLC Scanner 4 (CAMAG, Muttenz, Switzerland).

2.2. Media, agars and cultivation of the yeasts

Media and agars were composed as described in an earlier publication [8]: growth medium consisted of 6.8 g/L YNB, 1 g/L glucose, 170 mg/L L-lysine and 100 mg/L L-histidine. Test medium additionally contained 112 μM $CuSO_4$. Plate agar was composed of YNB (6.7 g/L), casamino acids (10 g/L), adenine (50 mg/L) and agar (20 g/L). Cultivation of yeasts was performed as described in [8]. Briefly, for overnight cultures, growth medium was inoculated with a colony from an agar plate and incubated overnight. Test cultures were prepared by centrifuging the required volume of overnight cultures and re-suspending the cell pellets in test medium. The cell number was measured with a TC20 automated cell counter (Bio-Rad Laboratories, Munich, Germany).

2.3. Substrate solution

Substrate dipping solutions with a concentration of 0.1 mg RGP/mL were prepared by diluting 200 μL of a stock solution of RGP (20 mg/mL in DMSO, stored at −20 °C) in 40 mL phosphate buffer consisting of 40.8 g/L KH_2PO_4, 42.6 g/L Na_2HPO_4, 1.2 g/L $MgSO_4 \cdot 7H_2O$, 3.7 g/L KCl (adjusted to pH 7 with 20% sodium hydroxide solution) [8].

2.4. Planar yeast estrogen screen (pYES)

HPTLC glass plates silica gel 60 RP-18 W were prewashed with acetone/water (9/1, v/v), heated for 30 min at 120 °C on the TLC Plate Heater III (CAMAG) and cooled down afterwards. To adjust the pH value of the plates to about 6.5, the plates were pretreated with a solution of sodium hydrogen carbonate (25 g/L, pH 6.4) [8]. Sample extracts were applied as 8 mm × 20 mm areas with an Automatic TLC Sampler 4 (ATS4, CAMAG) onto 10 × 10 cm HPTLC plates (15 mm from the lower edge, 12 mm from the left side, track distance set to automatic). The application zones were focused with acetonitrile up to 25 mm in a twin-trough chamber (10 cm × 10 cm, CAMAG). Chromatography was performed with a mixture of MeOH/H_2O (3/2, v/v) up to a migration distance of 70 mm using an Automatic Developing Chamber 2 (ADC2, CAMAG). Afterwards, yeast incubation (6–8 × 10^7 cells/mL, 4 h, 30 °C) and substrate incubations (RGP in 0.6 M phosphate buffer, 0.1 mg/mL, pH 7, 3 × 30 min, 37 °C) were performed as described in the earlier work [8]. HPTLC plate documentation was performed with a TLC Visualizer and a TLC Scanner 4 (both CAMAG). Plate images were captured under UV 254 nm, UV 366 nm and white light illumination, and the HPTLC plates were scanned in fluorescence mode at 550/ > 580 nm (tungsten lamp). TLC instruments were operated by the software winCATS, version 1.4.6 (CAMAG).

2.5. Samples and extraction

Hops pellet samples were obtained from the Distillery for Research and Training, University of Hohenheim, Germany. Two samples without further information about the hop variety were from Germany (hops A and B), one sample was from Great Britain (variety First Gold) and one from the USA (variety Galena). The hops pellets were ground in a mortar before extraction. The pestled hops sample (0.15 g) was extracted with ethanol (1 mL) by sonication for 10 min, followed by centrifugation at 13,000*g* for 5 min. The supernatant extract was evaporated in a nitrogen stream and the residue dissolved in 200 μL of ethanol. Subsequently, the extract was applied (4 μL) onto an HPTLC plate and subjected to pYES as described above (2.4 pYES). Additionally, the wort boiling part of the brewing process was simulated by cooking the pestled hops (0.15 g) in water (0.1 L) for one hour under constant stirring. In parallel, this process was also performed without heating. After centrifugation, the hops' remainders were extracted with ethanol as described above. The supernatant aqueous extract (1 mL) was extracted with diethyl ether (2 mL) by vortexing for 2 min [13]. The separated organic phase was evaporated by a nitrogen stream, and the residue was dissolved in 200 μL of ethanol. After the application of the extracts (4 μL of the hops' remainder extracts, 60 μL of the ether extracts of the supernatant water), pYES was performed as described above (2.4 pYES).

2.6. High-performance thin-layer chromatography–mass spectrometry

An ethanolic extract of the hops' remainder after boiling was applied onto an HPTLC plate (2-fold application of 4, 6, and 8 μL) that was cut into two sections after chromatography. One section of the plate was used for pYES, whereafter the coordinates of active zones were transferred to the other plate section used for mass spectrometry. Zones of interest (8 μL application) were eluted with the oval elution head of a TLC-MS interface (CAMAG) using methanol/ammonium formate buffer (10 mM, pH 4, 98:2, v/v [13]) and the eluate was transferred online at a flow rate of 0.2 mL/min (pump: Jasco PU-980, Groß-Umstadt, Germany) to a single quadrupole mass spectrometer (G1956B MSD, Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) interface. For negative ionization the following parameters were used: capillary voltage 3.0 kV, drying gas temperature 300 °C, drying gas flow rate 10 L/min, and nebulizer gas pressure 40 psig. Mass spectra in negative scan mode were recorded in a range of *m/z* 150 to *m/z* 450 with a fragmentor voltage of 100 V, gain 2.0, a step size of 0.1 and threshold 100. Data was recorded with the software ChemStation B.02.01 SR2 (Agilent Technologies).

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