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Fluorescent labeling of cranberry proanthocyanidins with 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein (DTAF)

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ABSTRACT

A novel methodology was developed to elucidate proanthocyanidins (PAC) interaction with extra-intestinal pathogenic *Escherichia coli* (ExPEC). PAC inhibit ExPEC invasion of epithelial cells and, therefore, may prevent transient gut colonization, conferring protection against subsequent extra-intestinal infections, such as urinary tract infections. Until now PAC have not been chemically labeled with fluorophores. In this work, cranberry PAC were labeled with 5-([4,6-dichlorotriazin-2-yl]amino) fluorescein (DTAF), detected by high-performance liquid chromatography with diode-array detection and characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). We report single and double fluorescent-labeled PAC with one or two chlorine atoms displaced from DTAF in alkaline pH via nucleophilic substitution. Fluorescent labeling was confirmed by fragmentation experiments using MALDI-TOF/TOF MS. Fluorescent labeled PAC were able to promote ExPEC agglutination when observed with fluorescence microscopy. DTAF tagged PAC may be used to trace the fate of PAC after they agglutinate ExPEC and follow PAC-ExPEC complexes in cell culture assays.

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1. Introduction

Cranberry proanthocyanidins (PAC) interact with extra-intestinal pathogenic *Escherichia coli* (ExPEC) inducing bacterial agglutination and reducing bacterial invasion in the gut (Feliciano, Meudt, Shanmuganayagam, Krueger, & Reed, 2013). Although previous studies demonstrated that cranberry PAC affect *E. coli* virulence factors (Johnson et al., 2008; Liu et al., 2008; Liu, Pinzón-Arango, Gallardo-Moreno, & Camesano, 2010), an understanding of the interaction between cranberry PAC and ExPEC is still lacking.

E. coli has been tagged with fluorescent probes (Loh & Ward, 2012) but a better insight on ExPEC-host cell interactions requires fluorescent labeled PAC. Cranberry PAC exhibit fluorescence but have a very narrow excitation and emission wavelength in the ultraviolet region, 276 and 310 nm, respectively (Adamson et al., 1999), and therefore cannot be used in fluorescence microscopy. The use of fluorescent probes to label PAC has not been described until now. On the contrary, fluorescence labeling of proteins has been widely used for cell biology and biochemistry applications (Toseland, 2013). Fluorescein isothyocianate (FITC), the most widely used fluorochrome, is reactive towards nucleophiles, such as amine groups in proteins (Blakeslee & Baines, 1976; Lalljie & Sandra, 1995) but does not react with molecules with multiple hydroxyl groups, such as in cranberry PAC.

5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF) has the advantage of reacting with amines (Blakeslee & Baines, 1976; Krahn, Bouten, van Tuijl, van Zandvoort, & Merkx, 2006; Liu, Hu, Ma, & Lu, 2004; Molina & Silva, 2002; Siegler, Sternson, & Stobaugh, 1989; Wadsworth & Sloboda, 1984; Wang, Li, Li, Hu, & Chen, 2006; Xiao et al., 2007) and hydroxyls in alkaline aqueous solutions (Abitbol, Palermo, Moran-Mirabal, & Cranston, 2013; Ahmed, Alexandridis, & Neelamegham, 2001; Schumann & Rentsch, 1998; Weyermann et al., 2004; Wicks & Li, 2004). The DTAF labeling reaction with hydroxyl groups follows a nucleophilic aromatic substitution by an addition–elimination pathway (Ahmed et al., 2001). In this work, we investigated the possibility

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of labeling PAC with DTAF after optimization of reaction parameters.

We report the detection of fluorescent-labeled PAC with DTAF using high-performance liquid chromatography with diode-array detection (HPLC-DAD), followed by structural confirmation with matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF MS). Fluorescent-labeled PAC were mixed with ExPEC and bacterial agglutination with emission of fluorescence was detected using fluorescence microscopy.

2. Materials and methods

2.1. Chemicals

Water, methanol, acetone (HPLC grade), acetic acid glacial, trifluoroacetic acid, sodium carbonate and sodium chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (200 proof) was obtained from Decon Labs Inc (King of Prussia, PA, USA). Triethylamine was purchased from Acros Organics (Geel, Belgium). Sterilized water, thyamin hydrochloride, 2,5-dihydroxybenzoic acid (DHB) and the Folin–Ciocalteu reagent were purchased from Sigma–Aldrich, (St. Louis, MO, USA). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein was obtained from Invitrogen (Carlsbad, CA, USA). HyClone Dulbecco's PBS without calcium and magnesium was obtained from Thermo Scientific (South Logan, UT, USA). Tryptose and dextrose were obtained from BD (Sparks, MD, USA).

2.2. Extraction and isolation of proanthocyanidins

Cranberry fruits *(Vaccinium macrocarpon* Ait. cv. 'Stevens') were obtained from the United Cranberry Growers Cooperative (Wausau, WI, USA). The fruits were homogenized to a fine powder by blending with liquid nitrogen. The fruit powder was stored at −80 °C until extraction.

The PAC extraction procedure was adapted from our previous work (Feliciano, Shea et al., 2012) using a total weight of 1314 g of cranberry powder (151.15 g of dry matter). For each 100 g of cranberry powder, 400 mL of 70% aqueous acetone were added and extracted in an ultrasonic bath for 15 min. The extract was centrifuged at 400*g* at 15 °C for 10 min and the supernatant was collected. The extraction was repeated two additional times and the supernatants combined. After filtration with cellulose paper, acetone was removed by evaporation under vacuum at 35 °C and the remaining suspension was solubilized in ethanol. The extract was then centrifuged at 13,416*g* at 0 °C for 10 min to eliminate insoluble material. The supernatants were used to isolate PAC by chromatography on Sephadex LH-20.

The ethanolic cranberry extract was loaded on glass columns (2.5 cm I.D. × 60 cm length, Kontes, Chromaflex) packed with Sephadex LH-20 that were previously swollen and washed in water and equilibrated with ethanol for 45 min at a flow rate of 4 mL/min (Feliciano et al., 2013). The column was eluted with ethanol, ethanol:methanol (1:1) and 80% aqueous acetone. The 80% aqueous acetone fraction was evaporated to dryness and dissolved in 67% aqueous methanol, and defined as "unlabeled proanthocyanidins" or UL-PAC. The extraction of PAC from 151.2 g of cranberry powder dry matter, derived from whole fruits, generated 2.43 g of PAC dry matter. The cranberry fruit had a moisture content of 88.5% and a PAC yield of 1.6%.

2.3. Synthesis of fluorescent labeled cranberry proanthocyanidins

UL-PAC (0.858 mL, 90.1 mg dry matter/mL) was evaporated and solubilized in 50 mL of 80% aqueous acetone under nitrogen. The pH of the solution was raised to ~10.5 with the addition of 0.8 mL of triethylamine (0.935 M in 80% aqueous acetone). DTAF (36 mg) was added to the solution and incubated at room temperature for 30 min with a magnetic stirrer. The reaction was quenched after 30 min with the addition of 0.050 mL of 8 M acetic acid. The acetone was removed by evaporation and the remaining water was diluted 1:1 with ethanol. This reaction mixture was loaded onto a column that contained 20 g of Sephadex LH-20 and was previously washed thoroughly with 400 mL of water and equilibrated with 100 mL of 50% aqueous ethanol. The column was eluted with 100 mL of 50% aqueous ethanol (eluate A), 500 mL of ethanol (eluate B) and 200 mL of 80% aqueous acetone. While eluate A and B were evaporated to dryness and solubilized in water with at least 50% of methanol, the 80% aqueous acetone fraction was evaporated to dryness and solubilized in 75% aqueous methanol and defined as "fluorescently labeled proanthocyanidins" or "FL-PAC".

2.4. Characterization of proanthocyanidins

2.4.1. High-performance liquid chromatography

The PAC content of UL-PAC and FL-PAC was quantified by the Folin–Ciocalteu method and results were expressed as gallic acid equivalents (GAE/mL). UL-PAC was diluted 1:10 in 50% aqueous methanol to a final concentration of 6.99 mg GAE/mL. Eluates A, B and FL-PAC were diluted with 50% aqueous methanol to a final concentration of 0.19 mg GAE/mL in order to monitor the reaction by HPLC. All samples were filtered with a 0.45 μm PTFE membrane before analysis by high-performance liquid chromatography with diode array detection (HPLC-DAD). Samples were analyzed on an Agilent 1100 HPLC (Agilent, Santa Clara, CA, USA) with an autosampler, a quaternary solvent pump and a photodiode array detector. Chromatography was performed with a Luna C_{18} column (5 μm, 10.0 × 250 mm) (Phenomenex, Torrance, CA, USA). The elution solvents were 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in methanol (Solvent B). The elution profile (flow rate of 2 mL/min) started at 10% solvent B and increased by 1% every min up to 80% of solvent B then ramped to 100% of solvent B in 1 min and held constant for 5 min. The gradient was reverted to 10% solvent B for 10 min to equilibrate the column. The elution was monitored at 280 and 443 nm, using Open LAB CDS ChemStation Edition C.01.05 software to collect and analyze chromatograms.

2.4.2. Matrix-assisted laser/desorption ionization time-of-flight

Previously developed MALDI-TOF MS methods (Feliciano, Krueger, Shanmuganayagam, Vestling, & Reed, 2012) were applied to characterize UL-PAC and FL-PAC. An aliquot of each fraction was evaporated to dryness and suspended in ethanol to a final concentration of approximately 20 mg GAE/mL. An aliquot (1.0 μL) of each fraction was mixed with 8 μL of DHB (50.0 mg/mL in ethanol) and placed in three different wells on the stainless steel MALDI target. Mass spectra were collected on a Bruker ULTRAFLEX-III™ MALDI-TOF/TOF (Billerica, MA, USA), equipped with a Smart Beam™, a two-stage gridless reflection, and a LIFT™ cell. Mass spectra acquired in positive reflection mode are the sum of spectra from different locations in each well with the deflection set at 550 and 1000 Da for UL-PAC and FL-PAC, respectively. MSMS spectra were collected using the LIFT™ cell. For UL-PAC between degree of polymerization (DP) 3 and 8, the monoisotopic ion was specified, and for FL-PAC between 1000 and 1500 Da, the most intense ions in each cluster were specified. Compass™ v1.3 software controlled the instrument. A standard PAC preparation was used for calibration as previously described (Feliciano, Shea et al., 2012). FlexControl and FlexAnalysis (Bruker Daltonik GmbH, Bremen, Germany,

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