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Isolation, analysis and structures of phototoxic fagopyrins from buckwheat

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ABSTRACT

Buckwheat products are commonly used in health foods and food supplements. However, public awareness regarding the presence of photodynamic naphthodianthrones fagopyrins that can cause photosensitization is low. At least two additional compounds with structures similar to that of fagopyrin are known to exist; however, the structures of these compounds have never been determined. In this work, we improved the extraction procedure and the chromatographic analysis of fagopyrins by developing a simple, sensitive and high-resolution high performance liquid chromatography (HPLC) analytical method using fluorescence detection. We observed at least six fagopyrin derivatives, which were isolated and characterized via UV–Vis absorption, NMR spectroscopy and mass spectrometry. We determined the structures of two new derivatives (fagopyrin A and fagopyrin E) and proved the existence of protofagopyrins that can transform into fagopyrins upon light exposure. Our methods complement the existing knowledge regarding fagopyrins and will allow for their further analysis, isolation and investigation of their biological activity.

1. Introduction

With the growing popularity of healthy diets, consumption of buckwheat products is increasing (Bonafaccia, Marocchini, & Kreft, 2003; Christa & Soral-Smietana, 2008). Buckwheat (*Fagopyrum* sp.) has been recognized as a potential anti-inflammatory agent due to its high polyphenol content, especially rutin (Kalinova & Vrchotova, 2009; Kreft, Knapp, & Kreft, 1999; Oomah & Mazza, 1996; Watanabe, 1998; Watanabe, Ohshita, & Tsushida, 1997). Those antioxidants affect blood vessels and can improve leg edema in patients with chronic venous insufficiency (Ihme et al., 1996; Wójcicki, Barcew-Wiszniewska, Samochowiec, & Rózewicka, 1995). The polyphenols contribute to the prophylaxis and treatment of metabolic syndromes, such as hypertension and diabetes (Archimowicz-Cyryłowska, Adamek, Droździk, Samochowiec, & Wójcicki, 1996; Kawa, Taylor, & Przybylski, 2003). High antioxidant activity is believed to inhibit both tumor growth (Chan, 2003) and carcinogenesis (Ishii et al., 2008). Buckwheat contains fagopyrins, phototoxic agents which can act as photosensitizers upon excitation with visible light causing fagopyrism (Hinneburg & Neubert, 2004), but these compounds have received little research attention. Symptomatically, fagopyrism is manifested as a skin irritation that occurs with sunlight exposure following the ingestion of large quantities of buckwheat. The phenomenon has primarily been observed in domestic animals, although rare human cases have also been reported. The pharmacologic properties of fagopyrin-rich buckwheat extracts have been discussed in several studies. The phototoxicity of the buckwheat herb was confirmed by *in vivo* tests on unpigmented rats and mice (Chick & Ellinger, 1941; Wender, Gortner, & Inman, 1943). A study of the phototoxic potential of buckwheat extracts on fibroblasts confirmed the role of irradiation using artificial sunlight. The light-dependent activity of the fagopyrins may also contribute to their potential use as sensitizers in photodynamic therapy (Ebermann, Alth, Kreitner, & Kubin, 1996). Fagopyrin-rich extracts exhibited antiproliferative activity through the fluorescent light and concentration-dependent inhibition of two receptor-associated proteins, tyrosine kinase and serine/threonine kinase (Samel, Donnella-Deana, & De Witte, 1996). The light-dependent antiviral activity of buckwheat extract was comparable to that of St. John's wort (*Hypericum perforatum* L.), which contains hypericin (Hagels, Wagenbreth, & Schilcher, 1995; Yip, 1993). Hypericin is a well-known phototoxic, antiviral and antibacterial agent whose properties are likely linked with an ability to form superoxide anions and singlet oxygen. Hypericin also contributes to the antidepressant

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and anti-inflammatory action of St. John's wort extracts (Butterweck, 2003; Kennedy & Wightman, 2011; Lakhan & Vieira, 2010; Prakash, Arulkumar, & Sabesan, 2010). Structurally, hypericin and fagopyrin are both naphthodianthrones and are thus expected to have similar activities. Their biosynthetic pathways are both presumably connected to phenol production through a polyketide pathway (Sytar, Brestic, & Rai, 2012). These data indicate the necessity for more detailed fagopyrin research, but a lack of good analytical methods presents an obstacle to this goal (Li & Zhang, 2001).

Several methods have been tested to isolate pure fagopyrins, obtain their mass and NMR spectra, and elucidate their structures. Fluorescent red crystals with at least two other similar substances of unknown structures have been isolated and named fagopyrins. The basic structure of fagopyrin was determined by observing the chemical transformations in purified buckwheat extracts using IR, UV–Vis and mass spectral analyses. The structure was found to contain a naphthodianthrone skeleton with two piperidines. Most of the data were compared with those from hypericin measurements (Brockmann, Weber, & Pampus, 1952; Eguchi, Anase, & Osuga, 2009; Samel et al., 1996; Wender et al., 1943). The NMR analyses were performed only on chemically modified fagopyrin tetrabenzoate and tetraacetate (Brockmann & Lackner, 1979). Despite many attempts, the successful separation, isolation and identification of the original fagopyrin derivatives have not yet been reported.

Eguchi et al. developed a high-performance liquid chromatography (HPLC) method with UV–Vis detection for determining the fagopyrin content. They obtained three chromatographic peaks thought to be fagopyrins. According to the known structures and transformations of hypericin derivatives, several forms of fagopyrin derivatives were postulated as pseudo-, proto- and protopseudo-forms (Eguchi et al., 2009). A pre-form called protofagopyrin transforms into fagopyrin in plant extracts under exposure to sunlight. The HPLC method was compared and found superior to the UV–Vis photometry method developed by Ožbolt, Kreft, Kreft, Germ, & Stibilj (2008). However, the chromatographic separation was unsatisfactory for further molecular characterization. This method did not utilize fagopyrin's characteristic fluorescence.

The current study aimed to determine the structures of fagopyrin and its derivatives isolated from buckwheat. We also sought to improve the isolation procedure and the resolution and sensitivity of the chromatographic analyses by employing specific fluorescence detection. The individual fagopyrin derivatives were characterized and new information regarding their structures was obtained via UV–Vis absorption, NMR spectroscopy and mass spectrometry.

2. Materials and methods

2.1. Plant material

The common buckwheat (*Fagopyrum esculentum* Moench) herb was harvested at flowering time and dried in the shade at room temperature. For some experiments, the flowers were separated from the rest of the herb.

2.2. Extraction of the fagopyrin for analytical purposes

Several different protocols were tested to optimize the analytical method and are described in the Results section. The optimal method was as follows: The sample (1.00 g) was macerated in acetone/water (9/1) (10 mL) for 24 h on a shaker (80 rpm, 37 °C). During that time, the sample was initially exposed to 30 min of sonication and later to six additional 5-min sonication treatments at equal intervals. Prior to each HPLC analysis, the sample was exposed to light for 1 h in a transparent HPLC vial to convert the protofagopyrins to fagopyrins.

2.3. Extraction of the fagopyrin for preparative purposes

The dry-milled buckwheat samples (5 g) were extracted with dichloromethane (1 L) to remove the chlorophyll. The remaining plant material was dried and extracted with 500 mL acetone/water (4/1). The acetone and water were evaporated and the residue was washed several times with water and 50% ethanol to remove the yellow flavonoid fraction. The dark red residue was dissolved in methanol/DMSO (1/1) and subjected to HPLC separation. The individual peaks were collected and evaporated under reduced pressure. The dried compounds, strongly bound to the glass, were washed with water and dried under nitrogen prior to being dissolved in the NMR solvent.

2.4. Chromatographic analysis

The HPLC system (Shimadzu Prominence) consisted of a system controller (CBM-20A), a column oven (CPO-20AC) and a solvent delivery pump with a degasser (DGU-20A5) connected to a refrigerated autosampler (SIL-20AC) with a photodiode array (PDA) detector (SPD-M20A) that monitored the wavelengths 190–800 nm and a fluorescence detector (LC-20AD XR, excitation wavelength = 330 nm and emission wavelength = 590 nm). The responses of the detectors were recorded using LC Solution software version 1.24 SP1. The chromatography was performed at 40 °C and a flow rate of 2 mL min^{-1} using a Phenomenex Kinetex® XB-C18 column (10 cm × 4.6 mm I.D., 2.7 μm particle size). Other columns were also tested, but were found inferior (Ascentis® Express C18, 10 × 4.6 mm, 2.7 μm; Chromolith® SemiPrep 100–10 mm, Rp-C18; Kinetex™ 2.6 μ XB-C18 100A, 100 × 4.6 mm).

The following gradient method using water (solvent A) and acetonitrile (solvent B), both containing 0.1% trifluoroacetic acid, was utilized: 0.01–0.20 min 2% B, 0.20–0.21 min 2–48% B, 0.21–26.00 min 48–52% B, 26.00–26.10 min 52–100% B, 26.10–34 min 100% B, 34.10–38.00 min 2% B. The addition of acetic acid and formic acid instead of trifluoroacetic acid was also investigated, but the performance with these acids was found to be inferior.

2.5. Mass spectrometric analysis

The chromatographic separation was performed on a Waters Acquity ultra-performance liquid chromatograph® (Waters Corp., Milford, MA, USA) with a column identical to that used for the quantitative HPLC analysis. The following gradient method was utilized with MilliQ water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B): 0–1 min 0% B, 1–48 min 48–52% B, 104–120 min 100% B with an injection volume of 10 μL. The flow rate was 0.5 mL min^{-1}, and the column temperature was maintained at 40 °C. The LC system was interfaced with a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-ToF Premier, Waters, Milford, MA, USA). The compounds were analyzed under positive (ESI(+)) and negative (ESI(−)) ion conditions. The capillary voltage was set at 3.0 kV, while the sampling cone voltage was 20 V. The source and desolvation temperatures were 120 and 200 °C, respectively. The nitrogen desolvation gas flow rate was 500 L h^{-1}. The acquisition range was between *m/z* 50 and 1000 with argon serving as the collision gas at a pressure of 4.5×10^{-3} mbar in the T-wave collision cell. The MS/MS experiments were performed using a collision energy of 30 eV to generate the product ion spectra that provided the best structural information. The data were collected in centroid mode, with a scan accumulation time of 0.2 s and an interscan delay of 0.025 s. The data station utilized the MassLynx v4.1 operating