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Antioxidant-guided isolation and mass spectrometric identification of the major polyphenols in barley (*Hordeum vulgare*) grain

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ABSTRACT

In the present study, the relative contribution of individual/classes of polyphenols in barley, to its antioxidant properties, was evaluated. Flash chromatography was used to fractionate the total polyphenol extract of Irish barley cultivar 'Irina', and fractions with highest antioxidant properties were identified using total phenolic content and three *in vitro* antioxidant assays: DPPH, FRAP, and ORAC. Flavanols (catechin, procyanidin B, prodelphinidin B, procyanidin C) and a novel substituted flavanol (catechin dihexoside, $C_{27}H_{33}O_{16}^-$, *m/z* 613.17), were identified as constituents of the fraction with highest antioxidant capacity. Upon identification of phenolics in the other active fractions, the order of most potent contributors to observed antioxidant capacity of barley extract were, flavanols > flavonols (quercetin) > hydroxycinnamic acids (ferulic, caffeic, coumaric acids). The most abundant polyphenol in the overall extract was ferulic acid (277.7 μg/g dw barley), followed by procyanidin B (73.7 μg/g dw barley).

1. Introduction

The role of dietary polyphenols in human health has been explored to a great extent in the past few decades due to their ability to reduce oxidative stress, induced by the generation of harmful free oxygen radicals in the body. Uncontrolled oxidative stress causes damage to major biomolecules, including the proteins, lipids and DNA, and may be critical to the aetiology of a number of degenerative diseases, such as cancer (Thanan et al., 2014), atherosclerosis (Li, Horke, & Förstermann, 2014) and other inflammatory disorders (Tak, Zvaifler, Green, & Firestein, 2000; Rezaie, Parker, & Abdollahi, 2007). Recently, oxidative stress has also been associated with the neurodegenerative disorder, Alzheimer's disease (Wang et al., 2014). In addition to their possible health benefits, antioxidants can be used to retard oxidative deterioration of lipids in foods which lead to the development of rancid off-flavours. Naturally occurring antioxidants, such as phenolic compounds in food sources, are often preferred to their synthetic counterparts because of consumer concerns associated with health and safety of synthetic antioxidants (Branen, 1975).

The most well-known sources of polyphenols include green tea, fruits, vegetables, beans, and cereals (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Compelling evidence on the *in vitro* and *in vivo* antioxidant activities of dietary polyphenols from a number of sources has been presented (Serafini, Ghiselli, & Ferro-Luzzi, 1996; Nigdikar, Williams, Griffin, & Howard, 1998; Jensen et al., 2008). Whole grains and cereals have also been well explored in the last two decades, and in many cases, their benefits on humans have been linked to their content of phenolic compounds (Slavin, 2004; Dykes & Rooney, 2007). Barley is the most abundantly grown cereal in Ireland that finds maximum applications in the brewing industry or as animal feed, while only about 2% of the grain is used for food directly (Sullivan, Arendt, & Gallagher, 2013). The scope of increasing the use of barley and its products in food applications warrants exploiting the potential of Irish-grown barley, with respect to its associated health benefits.

The purported health benefits of barley are often linked to its antioxidant properties, which are largely derived from its polyphenolic content (Goupy, Hugues, Boivin, & Amiot, 1999; Bonoli,

Abbreviations: FCR, Folin-Ciocalteu reagent; HCl, hydrochloric acid; EA, ethyl acetate; H_2SO_4, sulphuric acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, 2.4.6-tri (2-pyridyl)-s-triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TPC, total phenolic content; FRAP, ferric ion reducing antioxidant power; ORAC, oxygen radical absorbance capacity; AAPH, 2,2′-Azobis (2-amidinopropane) dihydrochloride; ET, electron transfer; HAT, hydrogen atom transfer; UHPLC-MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry; MRM, multiple reaction monitoring; Q-TOF, quadrupole Time of Flight; RDA, retro-Diels Alder; CID, collision induced dissociation.

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Marconi, & Caboni, 2004; Holtekjølen, Kinitz, & Knutsen, 2006). Phenolic compounds in barley exist in so-called free, soluble conjugated, and insoluble bound forms. The insoluble bound forms of phenolic acids are linked by ester or ether linkages to the cell wall material of the grain and require acid, alkaline or enzymatic hydrolysis for their release (Gangopadhyay, Hossain, Rai, & Brunton, 2015). In contrast, free polyphenols can be extracted using solvents, such as methanol, ethanol and acetone. The majority of the free phenolics in barley are flavanols that are usually found in their monomeric form as catechin and epicatechin, or in their polymeric form as proanthocyanidins (Bonoli et al., 2004; Holtekjølen et al., 2006). The bound phenolics in barley include phenolic acids particularly hydroxycinnamic acids, such as ferulic acid, which can also exist in its dimeric or trimeric form. Coumaric acid and caffeic acid are also often reported as part of the bound fraction of barley grains (Bonoli et al., 2004; Holtekjølen et al., 2006; Verardo, Bonoli, Marconi, & Caboni, 2008).

Although previous studies have given meaningful insights into the different polyphenols present in barley extracts, the identities of the individual phenolic compounds which are strongest contributors to the observed antioxidant capacity of barley are still unknown. Antioxidant-guided identification is a method of chromatographically fractionating a sample extract, following which the most antioxidant-active fractions are chosen, and the identity of the predominant contributors to the observed antioxidant capacity of the sample are established. The objective of the current study was to employ a flash chromatography fractionation of the barley grain extract followed by antioxidant-guided identification of polyphenols in the fractions.

2. Materials and methods

2.1. Materials

Hulled Irish spring barley cultivar 'Irina' from the 2013 harvest was provided by Seedtech (Waterford, Ireland). Whole barley grains were milled using a Perten Lab mill 3100 (Perten Instruments, AB, Kungens Kurva, Sweden). HPLC-grade ethyl acetate, methanol, hexane and water were purchased from Sigma Aldrich, Wicklow, Ireland. The polyphenols, caffeic acid, *p*-coumaric acid, ferulic acid, catechin and quercetin, were purchased from Sigma-Aldrich, Wicklow, Ireland. The polyphenol standard of procyanidin B1 was purchased from Extrasynthèse, Lyon, France. The purity of the standards and solvents were in the range of 95–99%. Folin-Ciocalteu reagent (FCR), gallic acid, sodium carbonate (Na_2CO_3), sodium acetate anhydrous, α-amylase, cellulase, 98% sulfuric acid (H_2SO_4), ferric chloride hexahydrate, hydrochloric acid (HCl), 2,2-diphenylpicrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Wicklow, Ireland. The Oxiselect ORAC assay kit was purchased from Cell BioLabs, Inc., San Diego, CA). Fluorescein probe and the radical generator (AAPH) were provided as a part of the ORAC kit.

2.2. Extraction of free and bound phenolics

An illustration of the workflow employed for antioxidant capacity-guided isolation and identification of phenolics in barley extract and its fractions is shown in Fig. 1. Extraction of free (unbound) phenolics from milled barley was carried out using the conditions optimised by Madhujith and Shahidi (2006). This involved sequentially extracting milled barley (100 g) three times in a shaker set at 60.5 °C. The extraction was carried out using 80.2% aqueous methanol for 38.3 min at a solids to solvent ratio of 1:10 (*w/v*) per extraction. The extracts were centrifuged at 5000 rpm for 15 min, and the obtained supernatant (1, 2 and 3), after each extraction, was filtered through a Büchner funnel (pore size ~ 1 μm), while the corresponding residue was used as a substrate for the next round of extraction. The pooled and filtered supernatants were defatted using hexane at a ratio 2:1 (*v/v*). The methanolic phase containing the free phenolics was dried immediately using rotary evaporation (Heidolph, Schwabach Germany). The residue from the third round of extractions of the free phenolics was used for the extraction of bound polyphenols using an acid, α-amylase, and cellulase hydrolysis according to the method of Yu, Vasanthan, and Temelli (2001), with some modifications. The acid hydrolysis step was carried out by mixing the residue with 1 L of 0.1 M H_2SO_4, and heating at 85 °C for 1 h. The sample was cooled for 10 min in an ice-water bath prior to the addition of 200 mL of 2.5 M aqueous sodium acetate solution containing 2% (*w/v*) α-amylase and incubated at 30 °C for 1 h. Following this, 100 mL of a 0.1 M aqueous sodium acetate solution containing 2% cellulase were added, and the sample was further incubated for 10 h at 30 °C. Upon centrifugation at 5000 rpm for 10 min, the obtained supernatant of the aqueous extract was filtered through a Büchner funnel. The filtered extract was defatted using hexane (2:1 v/v), after which the extract was subjected to a liquid–liquid partitioning using an equal volume of ethyl acetate. The ethyl acetate phase containing the bound phenolics from barley was dried using rotary evaporation. The total phenol content of the dried extracts was calculated and the dried extracts were stored at −20 °C until further use.

2.3. Fractionation of the barley polyphenols using reversed-phase flash chromatography

Prior to flash chromatography, the dried extracts of free and bound phenolics were each dissolved in minimal amounts of 80% methanol and mixed with each other to give the total pooled phenol extract from barley. About 1 g of the dry total polyphenol extract was resuspended in minimal amount (approximately 10 mL) of 80% methanol and fractionated on a Varian IntelliFlash flash chromatography system (Model 310). The column used for flash chromatography was a reversed-phase Telos C_{18} column with a sorbent mass of 140 g and an average particle size of 40–60 μm. A binary solvent system consisting of water plus 0.5% formic acid (mobile phase **A**) and acetonitrile plus 0.5% formic acid (mobile phase **B**) was used as the mobile phase. A stepwise gradient (0% **B** for 5 min, 10% **B** from 5 to 10 min, 20% **B** from 10 to 15 min, 30% **B** from 15 to 20 min, 80% **B** from 20 to 25 min, and 100% **B** from 25 to 35 min) at a flow rate of 40 mL/min was employed to separate the polyphenols of the pooled extract (Fig. 2). Fractions were collected at a time interval of 1.0 min over 35 min, resulting in 35 fractions. The eluting fractions were monitored at the wavelengths of 280 and 320 nm. As no visible peaks were detected on the chromatogram in the last 5 fractions, only the first 30 fractions were assayed for antioxidant capacities.

2.4. Determination of TPC and antioxidant capacities of the flash chromatography fractions

The total phenolic content (TPC) of the fractions was determined by Folin-Ciocalteu method and the antioxidant capacities were tested using two types of *in vitro* assays – a) single electron transfer (ET) reaction-based assays such as ferric ion reducing antioxidant power (FRAP), and DPPH b) hydrogen atom transfer (HAT) reaction-based assay, which included the oxygen radical absorbance capacity (ORAC) assay. Prior to the assays, the fractions were dried, and redissolved in 40 mL methanol. The experimental procedures of TPC and the two ET reaction-based assays (DPPH,