Phytochemistry 122 (2016) 65–75

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Compositional changes in (iso)flavonoids and estrogenic activity of three edible *Lupinus* species by germination and *Rhizopus*-elicitation

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ARTICLE INFO

Article history:
Received 3 August 2015
Received in revised form 16 November 2015
Accepted 22 December 2015
Available online 31 December 2015

Keywords:
Lupinus luteus
Lupinus albus
Lupinus angustifolius
Fabaceae
Genistein
2′-Hydroxygenistein
Mass spectrometry
Prenylation
Deglycosylation

ABSTRACT

The effects of germination and elicitation on (iso)flavonoid composition of extracts from three edible lupine species (*Lupinus luteus*, *Lupinus albus*, *Lupinus angustifolius*) were determined by RP-UHPLC–MS^n. The total (iso)flavonoid content of lupine increased over 10-fold upon germination, with the total content and composition of isoflavonoids more affected than those of flavonoids. Glycosylated isoflavones were the most predominant compounds found in lupine seedlings. Lesser amounts of isoflavone aglycones, including prenylated ones, were also accumulated. Elicitation with *Rhizopus oryzae*, in addition to germination, raised the content of isoflavonoids further: the total content of 2′-hydroxygenistein derivatives was increased considerably, without increasing that of genistein derivatives. Elicitation by fungus triggered prenylation of isoflavonoids, especially of the 2′-hydroxygenistein derivatives. The preferred positions of prenylation differed among the three lupine species. The change in isoflavone composition increased the agonistic activity of the extracts towards the human estrogen receptors, whereas no antagonistic activity was observed.

1. Introduction

Isoflavonoids have been associated with several health-promoting effects, including reduced risks of various cancers and alleviating effects of hormone replacement therapy (Boué et al., 2011; Birt et al., 2001; Barnes et al., 1994). These effects are (partially) exerted by binding of isoflavonoids to human estrogen receptors (hER) (Simons et al., 2012). Previously, we have developed an effective method to elevate the isoflavonoid content of soybeans by performing germination and fungal elicitation simultaneously (Aisyah et al., 2013). The treatment increased the total isoflavonoid content of soybeans by up to 2-fold, accompanied by compositional changes. The total amount of prenylated isoflavonoids was boosted up to 13-fold. As a result, up to 50% (wt/wt) of total isoflavonoids were prenylated pterocarpans, i.e. glyceollins (Aisyah et al., 2013). Glyceollin I, a major prenylated pterocarpan in elicited soybean, has been suggested as a novel therapeutic agent for hormone dependent tumors (Zimmermann et al., 2010).

Lupine (*Lupinus*) is a genus of the legume family, consisting of around 200–400 species (Williams et al., 1983). Contrary to most genera of legumes, roots and leaves of lupine can produce a variety of prenylated isoflavonoids in addition to glycosylated ones and aglycones (Harborne et al., 1976; Tahara et al., 1984). Further investigation on the ability of lupine to generate defense metabolites upon stress showed that lupine failed to induce isoflavonoids other than the constitutive ones (Harborne et al., 1976). Nevertheless, it has been observed that the isoflavonoid content of *Lupinus angustifolius* can be boosted in response to fungal infection (Wojakowska et al., 2013a). Moreover, fungal infection is known to increase the ratio of aglycones to glycosylated isoflavonoids of *Lupinus albus*, which was linked to an increase of β-glucosidase activity (Príslewska et al., 2002; Rybus-Zając and Kozłowska, 1996).

Contrary to studies on the content of isoflavonoids in leaf and root parts, the induction of isoflavonoids in lupine seedlings has not been extensively investigated. Furthermore, most studies focused on aglycones (Wojtaszek and Stobiecki, 1997). Additional to soybean, germinated or elicited lupine seeds might be a source of bioactive isoflavonoids. Hence, in the present study, the seeds of three edible lupine species, *Lupinus luteus*, *L. albus* and *L. angustifolius*, were subjected to the process of simultaneous germination

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http://dx.doi.org/10.1016/j.phytochem.2015.12.015
0031-9422/

and elicitation by fungus, which has been successfully applied to soybean seeds previously (Aisyah et al., 2013; Simons et al., 2011a,b). It is hypothesized that isoflavonoid content and molecular diversity of isoflavonoids in lupine seedlings, as well as the estrogenic potential of lupine seedling extracts, will change upon the treatment. Thereby, it will provide novel lead molecules for therapeutic purposes when compared to extracts obtained from soybean seedlings. The compositional changes during treatment were monitored by LC–MS/MS analysis with emphasis on prenylated isoflavonoids. For this, the current diagnostic tools for characterizing prenylation of isoflavonoids in complex extracts were extended using MS/MS fragmentation data (Simons et al., 2011a; Tahara et al., 1985; Xu et al., 2012).

2. Results

2.1. Chromatographic profiling of different lupine extracts

UHPLC-UV analysis of the *L. albus* extract showed that the extract from untreated seeds contained only a single peak (Fig. 1A), whereas both germination and elicitation by fungus generated an array of compounds (Fig. 1B and C). The untreated seeds of two other *Lupinus* species contained two peaks, one of which was eluted at the same retention time as that of *L. albus* (data not shown). Both other species showed a similar response to the treatments (germination and elicitation) as *L. albus*, although they accumulated different sets of compounds (Fig. 1D and E). In total, sixty-one peaks were used for further analysis. Within each chromatogram, the peaks analyzed represented more than 95% of the total UV response at 260 nm of the chromatogram. The (iso)flavonoids tentatively annotated belonged to the flavone, flavanone, isoflavone and coumaronochromone subclasses. The molecules were present in aglyconic, glycosylated and prenylated forms. The annotation of aglycones and glycosylated derivatives was based on comparison of spectral data obtained from UHPLC-UV–ESI-MS/MS (including fragmentation patterns) (Table S1) with literature data (Bednarek et al., 2003; Cuyckens and Claeys, 2004; Frański et al., 1999a,b; Kachlicki et al., 2005; Kuhn et al., 2003; Muth et al., 2008; Stobiecki et al., 1999; Veitch, 2009, 2013; Wojakowska et al., 2013b). Spectral analysis of LC–MS/MS data of the majority of prenylated derivatives, not previously described in the literature, is elaborated upon in the present study.

2.2. C- and O-glycosides of (iso)flavonoids

Twenty-seven non-prenylated (iso)flavonoids glycosides were tentatively annotated in treated *Lupinus*, comprising the flavone, flavanone and isoflavone subclasses. The different subclasses were discriminated on the basis of UV absorption. Typical λ_{max} values of 270 (±5) and 330–365 nm for flavones, 290 (±5) nm for flavanones, and 260 (±5) nm for isoflavones were observed (Marston and Hostettmann, 2006). The glycosylated derivatives had one to three glycosyl residues, some of which were malonylated. The glycosyl residues found previously in lupine were mainly glucosyl, xylosyl, and rhamnosyl, attached to hydroxyl groups or directly to a *C*-atom (Wojakowska et al., 2013b). In the present study, the *C*- and *O*-glycosylated (iso)flavonoids were distinguished by their characteristic fragmentation patterns resulting from the cleavage of the sugar moieties in MS^2 (Kachlicki et al., 2008, 2005). Neutral losses of 162/146/132 Da are ascribed to *O*-glucoside/*O*-rhamnoside/*O*-xyloside residues, respectively, whereas neutral losses of 90 and 120 Da are ascribed to *C*-glucosides of (iso)flavonoids in PI or NI mode (Ferreres et al., 2007; Kachlicki et al., 2008, 2005). Moreover, neutral losses of 150/164/180 Da in NI mode are attributed to *O*,*C*-diglycosides, namely *O*-xylosyl-*C*-glucosyl/*O*-rhamnosyl-*C*-glucosyl/*O*-glucosyl-*C*-glucosyl(iso)flavonoids, respectively (Ferreres et al., 2007). A high relative abundance of these neutral losses was ascribed to the attachment of one of these glycosyl residues at the 2″-OH of the *C*-glucosyl residue (Ferreres et al., 2007). In addition, a malonyl group was identified by neutral losses of 44 or 86 Da, in NI or PI mode, respectively (Muth et al., 2008; Wojakowska et al., 2013b).

2.3. Prenyl configuration of isoflavonoids in extracts of lupine seedlings

Twenty-five prenylated isoflavonoids were found in treated lupines, including prenylated isoflavonoid glycosides. The UV spectra indicated that most of the prenylated derivatives were isoflavones. Two compounds were tentatively annotated as prenylated coumaronochromones (**58** and **59**), which were distinguished from isoflavones by their UV spectrum, exhibiting λ_{max} values of around 257 (±2), 284 (±2) (*sh*), and 338 (±3) nm (Tahara et al., 1985). The prenyl group can be attached in different configurations to the A- and/or B-rings (Table 1). A neutral loss of 56 Da (C_4H_8) was used to distinguish a prenyl chain from a ring-closed prenyl in PI mode (Simons et al., 2009; Xu et al., 2012). Major neutral losses of 42 Da (C_3H_6) and, to a lesser extent, 60 Da ($C_3H_6 + H_2O$), 54 Da (C_4H_6) and 15 Da (CH_3) were used to annotate both 2,2-dimethylpyran and 2-isopropenyldihydrofuran rings (Xu et al., 2012). Neutral losses of 18 Da (H_2O) and 72 Da (C_4H_8O) were indicative of a 2-(1-hydroxy-1-methylethyl)-dihydrofuran ring (Xu et al., 2012). Based on these neutral losses, peaks **41**, **45**, **47**, **51**–**55** and **57**–**59** were classified as prenyl chain isoflavonoid derivatives. It was impossible to distinguish between isoflavonoid isomers with a pyran or furan substituent by mass spectrometry, as both of them provided almost the same fragmentation patterns. Nevertheless, these isomers were tentatively annotated on the basis of the literature, accounting for their elution behavior, their UV maxima, and their abundance in previous studies (Hashidoko et al., 1986; Stobiecki et al., 1999; Tahara et al., 1986, 1984, 1994, 1989). Peaks **42** and **48**–**50** were assigned as 2,2-dimethylpyran isoflavonoids, whereas **33** and **46** were assigned as 2-isopropenyl dihydrofuran and 2-(1-hydroxy-1-methylethyl)-dihydrofuran isoflavonoid, respectively. Moreover, lupine generated diprenylated isoflavonoids as well. The fragmentation behavior of diprenylated isoflavonoid was similar to that of monoprenylated isoflavonoid, only the diagnostic losses occurred twice: in MS^2 and MS^3 (Takayama et al., 1992). As a result, peaks **56** and **61** were annotated as isoflavonoids containing two prenyl chains, whereas peak **60** was assigned as an isoflavonoid containing a prenyl chain and a pyran-ring. In addition, five out of twenty-five prenylated isoflavonoids were in *O*-glycosylated form (peaks **13**, **26**, **30**, **34**, and **35**), no *C*-glycosylated form was found. This is consistent with the fact that prenylation and *C*-glycosylation can occur at the same positions. The type of prenyl attached to these glycosides of prenylated isoflavonoids can be determined from fragmentation of the aglycone product ions in either in MS^3 or MS^4. As a result, all glycosides of prenylated isoflavonoids were annotated as isoflavonoids containing a prenyl chain.

2.4. A- or B-ring prenylation in isoflavonoids

Analysis of *retro*-Diels-Alder (RDA) fragment ions in PI mode from isoflavone isomers was used to determine the position of the prenyl substituent (A- or B-ring) (Cuyckens and Claeys, 2004). RDA fragments diagnostic for A- or B-ring prenylation with a prenyl chain were obtained upon fragmentation of the ion $[M+H-C_4H_8]^+$ in MS^3 (Fig. 2A and B). The diagnostic fragments $^{1,3}A^+$-C_4H_8 and $^{1,3}B^+$-C_4H_8 still contained one carbon reminiscent of the prenyl chain (Hashidoko et al., 1986; Xu et al., 2012). This fragmentation behavior was confirmed with two authentic