1 Carbohydrate composition of mature and immature faba bean seeds
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Abstract

Faba bean is a valuable pulse crop for human consumption. The low molecular weight carbohydrates (LMWC): glucose, fructose, sucrose (GFS), raffinose, stachyose, and verbascose (RFO- raffinose family oligosaccharides) in faba bean seeds contribute to the flavor and prebiotic nature of this edible bean. Understanding the variation of these compounds across the species would aid plant breeders in their selection efforts to release improved varieties. Therefore, this study was conducted to quantify LMWCs from a diverse collection of faba bean germplasm. The LMWCs of mature and immature seed from 40 faba bean populations across a range of seed sizes (26.2-172.0 g·100 seed⁻¹) were quantified with an Agilent 1260 Infinity LC (size exclusion chromatography/gel permeation chromatography) system with refractive index detection. Sucrose was the predominant constituent LMWC of immature seeds ranging from 5.9 to 22.6% DW for cotyledons and 6.7 to 16.7% DW for seed coats, while total RFO averaged <1% DW across populations. The sucrose content of mature seeds was relatively stable across population with a mean of 2.4% DW, while RFO content ranged from 2.5 to 7.5% DW. The apparent positive relationship between seed size and GFS of immature seed and sucrose and RFO of mature seed indicates that selection for seed size may also affect LMWC concentration.

Keywords: *Vicia faba*, faba bean (fava bean); raffinose-family oligosaccharides; food analysis; food composition; nutritional quality; high performance liquid chromatography

Introduction

Faba bean (*Vicia faba* L.) is a versatile cool-season pulse crop utilized for human and animal consumption (Crépon et al., 2010). There are three main botanical varieties of faba bean: var. minor ($<40 \text{ g} \cdot 100 \text{ seed}^{-1}$), var. equina ($40\text{-}80 \text{ g} \cdot 100 \text{ seed}^{-1}$), and var. major ($>80 \text{ g} \cdot 100 \text{ seed}^{-1}$) (Duc, 1997). Minor and equina types are mainly utilized as mature dry seeds, while immature seed of var. major is most often consumed as a fresh vegetable (Hawtin and Hebblethwaite, 1983; Baginsky et al., 2013).

The main energy components and therefor value of mature faba bean seed is most often attributed to starch ($\sim 40\%$ DW) and protein ($\sim 30\%$ DW) contents (Pritchard et al., 1973; Guillon and Champ, 2002). However, low molecular weight carbohydrates (LMWC): glucose, fructose, sucrose (GFS) and raffinose family oligosaccharides (RFO): raffinose, stachyose, and verbascose also contribute to seed quality (White, 1966; El-Shimi et al., 1980; Freijngel et al., 1997; Duc et al., 1999).

The majority of interest has been given to RFOs as they are presumed to be antinutritional (Frauen et al., 1984; Ruperez, 1998) or non-nutritional (Torress et al., 2012). However, alternative opinions suggest that these flatulence-causing carbohydrates may also serve as important health promoting prebiotics (Tomomatsu, 1994; Frias et al., 1996; Champ, 2002; Martinez-Villaluenga et al., 2008). Hayakawa et al. (1990) showed that low doses ($3 \text{ g} \cdot \text{day}^{-1}$) of purified RFO could increase bifidobacteria and Kozłowska (2001) suggested $0.1\text{-}0.3 \text{ g}$ of RFO per portion would not likely cause digestive issues.

Sucrose is another LMWC considered to be a critical component of many foods, as it enhances natural flavors and improves the characteristics of other flavoring ingredients (Grotz

and Munro, 2009). For example, genotypes of dry mature cowpeas with a sucrose content of 6% compared to 2% were consistently chosen for flavor (Hall et al., 2003). Furthermore, small additions of 5% and 10% sucrose to the fiber rich butterbean (*Phaseolus lunatus* L.) enhanced taste and acceptance (Vorster et al., 1987).

The monosaccharides fructose and glucose are generally negligible in mature faba bean seed, but sucrose content has a wide distribution and can range from 0.02% to 5.23% DW (Pritchard et al., 1973; Cerning et al., 1975; Frauen et al., 1984; Lattanzio et al., 1986; Quemener, 1988; Frias et al., 1996). Comparatively less information is available for the LMWC content of immature faba bean seeds (Lattanzio et al., 1986; Ziena et al., 1987; Frias et al., 1996; Weber et al., 1996) especially from a diverse panel of germplasm.

Screening faba bean germplasm for LMWC content will help to inform breeders in their efforts to improve the sweetness and perhaps flavor of mature and immature seeds, while minimizing RFO content. Identification of genotypes with these desirable seed quality traits would help to expand market potential. The purpose of this research was to quantify LMWCs from 40 faba bean populations varying in seed size. Large to small-seeded classes obtained from commercial sources and germplasm accessions served to broadly characterize LMWCs of faba bean.

Materials and Methods

Forty faba bean populations (commercially available cultivars, open pollinated heirlooms, germplasm accessions from the USDA-ARS Western Regional Plant Introduction Station, and a breeding line ‘HE-F₅’, derived from a cross between winter-hardy European cultivar ‘Hiverna/2’ and a large-seeded commercial cultivar ‘Extra Precoce Violetto’ at the F₅ generation) were included in this study (Supplemental table 1). A representative sample of air dried mature seeds and immature seeds were selected from field grown plants in 2013 at Washington State University’s Whitlow Farm (WF) in Pullman, WA (46°44’3.2”N-117°7’25.8”W) and dehulled.

Immature seeds were harvested during the late cotyledon stage from 30 to 50 days after flowering when the sucrose content is reported to be highest (Lattanzio et al., 1986; Heim et al., 1993; Borisjuk et al., 1995; Frias et al., 1996). Collected immature cotyledons were separated from the seed coat and percent moisture was determined once lyophilized. Mature seeds were dehulled and the hulls discarded, since they contained negligible amount of LMWC (data not shown).

LMWC extraction and analysis was followed according to Knudsen and Li (1991) using water as the mobile phase instead of 0.015 N Na₂SO₄. Briefly, triplicate 200 mg samples of finely ground flour were extracted with 4 mL of EtOH (50%, v/v) plus 1 mL of ribitol as an internal standard for 1 hr at room temperature with intermittent shaking. The supernatant was then decanted after centrifugation (3,000 g, 10 min @ 24°C) and the pellet was rinsed with 3 mL and then 2 mL of EtOH (50%, v/v) after a 5 min reflux cycle. The supernatants were pooled and diluted to 10 mL with EtOH (50%, v/v). An aliquot (1.0 mL) from this stock was diluted with an equal volume of EtOH (90%, v/v), stored at -20 °C for 1 hr and centrifuged (10,000 g for 10 min

@ 24°C) to precipitate proteins. The supernatant was dried at 25 °C (Thermo Scientific DNA 110 Savant™ SpeedVac™, Waltham, MA, U.S.A.) and redissolved in 1 mL of ultrapure water (EMD Milipore Co.; Billerica, MA, U.S.A.) for high performance liquid chromatography (HPLC) analysis.

An Agilent 1260 Infinity LC system (size exclusion chromatography/gel permeation chromatography) with refractive index detection (Agilent Technologies, Santa Clara, CA, U.S.A.) at 35 °C was used to quantify individual LMWCs. The mobile phase was water with a flow rate of 0.5 mL·min⁻¹. An Aminex HPX-N87 (300 x 7.8 mm) resin-based column (Bio-Rad Laboratories, Inc.; Hercules, CA, U.S.A.) in the sodium form (300 x 7.8mm) preceded by a Cation H Bio-Rad Micro-Guard column (304.6mm) was used to separate LMWCs isocratically at 80 °C. Injection volume of the sample was 20 µL and run time was 15 min.

Quantification and assignment of peaks was based on the peak areas and retention times of known standard curves for purified glucose (C.A.S. 50-99-7, purity ≥99.5%), sucrose (C.A.S. 57-50-1, purity ≥99.5%), fructose (C.A.S. 54-48-7, purity ≥99%), verbascose (C.A.S. 546-62-3, purity ≥ 97%), stachyose (C.A.S 54261-98-2, purity ≥98%), raffinose (C.A.S. 17629-30-0, purity ≥98%), and ribitol (C.A.S. 488-81-3, purity ≥99%) (Supplemental Fig. 1) (Sigma-Aldrich Co.; St. Louis, MO, U.S.A.). Linearity of analysis was assessed based on standard curves at concentrations between 80-120% of the working range in sample concentrations. The minimal detectable limit across analytes was between 0.05 and 0.1 ppm. The coefficient of determination between peak area (y) and the mass in mg of glucose (0.999), sucrose (0.9998), fructose (0.9997), stachyose (0.9998), raffinose (0.9998), and verbascose (0.9999) was high. Precision expressed as the percentage of relative standard deviation at each concentration for each analyte was <4% based on three injections (n=3) (data not shown). Accuracy and reproducibility of

detection was below 5% based on independent runs. All samples were chromatographed under the same conditions as the standards and presented on a percent dry weight (% DW) basis.

LMWC contents of mature and immature seeds were compiled and analyzed ($p \leq 0.05$) using an ANOVA and Tukey's honest significant difference test with PROC MIXED (SAS, 2008). Analyses were conducted according to a completely randomized design with three biological replications and standard error bars are presented. Significant entry differences were assessed once significant seed type (mature seed, immature seed coat, and immature cotyledon) and seed weight class (small: $<41 \text{ g} \cdot 100 \text{ seeds}^{-1}$, medium: $42\text{-}85 \text{ g} \cdot 100 \text{ seeds}^{-1}$, and large: $>85 \text{ g} \cdot 100 \text{ seeds}^{-1}$) category differences were identified.

Results and Discussion

The current HPLC protocol worked very well to quantify individual LMWCs of faba bean seeds. The use of water as a mobile phase had the benefit of eliminating toxic waste disposal and provided simplicity and speed, however in this study it occasionally failed to fully resolve baselines in the mature seed for stachyose and verbascose (Supplemental Fig. 2). The retention time for each sugar was in most cases the same as that of the standard, but occasionally varied (0.2-0.4 sec) for fructose and constituent RFOs.

The LMWC elution patterns (Supplemental Fig. 2) and dry weight concentrations (Table 1) were characteristic for each sample tissue. This was expected based on the invertase control hypothesis of legume seed development (Weber et al., 1997); where cell wall invertase activity within the seed coat is related to growth and a high hexose state, whereas increasing sucrose synthase activity marks the transition from growth to storage product synthesis and maturation.

The accumulation of RFOs and concomitant decline in sucrose concentration marks this transition from dry weight accumulation to desiccation and a mature quiescent state (Obendorf and Górecki, 2012).

As expected, RFOs and sucrose predominated in mature seed, while sucrose constituted the major LMWC of immature seed. The primary RFOs of mature seed averaged across populations were verbascose (2.4% DW) and stachyose (1.9% DW), in agreement with past reports (Lattanzio et al., 1986; Quemener, 1988; Dini et al., 1989). Sucrose was highest for immature cotyledon (13.4% DW) and seed coat (8.0% DW) tissues and lowest for mature seed (2.5% DW). In general, the sucrose content of the var. major immature (15.7% DW) and mature (3.0% DW) cotyledons were 20% to 30% higher than either of the var. equina or var. minor classes. These results are consistent with others that have reported larger seeds generally have higher sucrose values than genotypes with smaller seeds (Cerning et al., 1975; Barratt et al., 1982; Lattanzio et al., 1986).

For simplicity total glucose, fructose, and sucrose (GFS) from immature seed (Fig. 1) and sucrose and raffinose, verbascose, and stachyose (RFOs) contents from mature seed (Fig.2) are presented for individual populations because of low concentrations (0.2% DW) in other tissues. The wider distribution for GFS content of immature seed (Fig. 1) as compared to sucrose or RFOs of mature seed (Fig. 2) across individual populations may be, at least in part, the result of variable seed maturation shown as moisture content of harvested immature seed (Supplemental Table 1). However, previous reports have also documented that GFS can range from 7% to 15% DW for whole immature faba bean seeds across populations (Lattanzio et al., 1986; Frias et al., 1996) and RFOs can range from 1.1% to 6.2% DW for mature seed (Lattanzio et al., 1986;

Quemener, 1988; Dini et al., 1989; Frias et al., 1996; Kozłowska, 2001; Guillon and Champ, 2002).

Few studies have investigated the LMWC content of immature faba bean seed coats. Weber et al. (1995) reported immature seed coat sucrose concentrations between 60-80 $\mu\text{mole}\cdot\text{g}^{-1}$ (FW), equivalent to about 2.5% (FW), which is similar to our dry weight data. Immature seed coats do appear to contribute significantly to the overall GFS content of immature seed (Fig. 1). This is especially true of smaller seeded populations, for example, the seed coat of ‘Scout’ comprised about 50% DW of the whole immature seed and had a similar moisture content as the cotyledon (Table 1). With both tissues having GFS values of about 10% DW, the seed coat is a significant reservoir of GFS content.

The GFS content of immature cotyledon and seed coat tissues did not always align, however. For example, ‘Polar’ was amongst the highest in cotyledon GFS concentration, but amongst the lowest in seed coat concentration. Several large seeded types ($>100\text{ g}\cdot 100\text{seeds}^{-1}$) such as ‘Peruvian Red Cheek’, ‘Copper’, and ‘Superaguadulce Morocco’ did however have elevated levels of GFS for both seed coat and cotyledon and ‘Sweet Lorane’ (medium), ‘HE-F₅’ (large), and ‘PI 655348’ (small) had some of the lowest GFS values for both tissues. Notwithstanding, the apparent relationship between seed size and GFS content for immature cotyledon and seed coat (Fig. 3) and sucrose and RFO content of mature seeds (Fig. 4) should be studied further.

Assuming a genetic basis for each component LMWC, it may be possible to breed varieties with a desirable LMWC profile to meet the need of end users. Recent advances in breeding edamame type soybean can be attributed to the identification of several germplasm accessions with high sucrose, low raffinose, and low stachyose contents (Hou et al., 2009;

Mozzoni et al., 2013). Evaluation of immature cotyledons from large seeded germplasm for GFS content is expected to be most promising, since this class was on average 20% higher than medium and 30% higher than small seed categories. Based on the germplasm investigated, there may be a relationship between GFS content of immature seed (Fig. 1) and sucrose content of mature seed (Fig. 2)($r=0.32$; $p=0.0003$), which would aid characterization. However, excluding analysis of immature seeds should be avoided. Immature cotyledons from ‘Copper’ for example had one of the highest GFS contents measured at 24.9% DW, while the sucrose content of mature seed was midrange at only 2.2% DW.

The GFS content of immature cotyledons was weakly correlated with RFO content of mature seed ($r=0.27$; $p=0.002$). One particular commercial cultivar that did not fit this trend was De Monica, which had a relatively high GFS content of immature cotyledon (17.6% DW) and seed coat (14% DW), but low RFO content for mature seed (2.5% DW). Genotypes with high sucrose content at the fresh consumption stage and low RFO content as a pulse are well suited for a dual-purpose fresh and dry market. Mature seed from small seeded ‘Sweet Lorane’ and ‘Hiverna/2’ also had a low total RFO concentration, but contained low GFS concentration of immature seeds. A comparison of our results with past research indicates that none of the entries tested in this study had mature seeds with less RFO content than cv. Beryl (1.4% DW), a low RFO line identified by Lattanzio et al. (1986).

Based on the multiple interactions between LMWCs and their representative quantitative distributions, it appears that selection would be complicated without sophisticated breeding designs coupled with analytical instrumentation at developmental stages aligned with a particular end-use. Further research should, therefore, focus on elucidating the heritability of LMWCs of faba bean and its relationship with seed size, similar to that done in lentil (Frias et al., 1999).

Anecdotal, sucrose and total RFO content between the mature seed of ‘HE-F₅’ and the parental ‘Extra Precoce Violetto’ were similar, as well as the percent seed coat of immature seed from the ‘Hiverna/2’ parent. However, the concentration of GFS between immature cotyledons of ‘HE-F₅’ and either parent was not clearly distinguishable.

Conclusion

This preliminary study of LMWCs from a broad collection of 40 faba bean entries demonstrates that there exists a wide variation of GFS and RFO contents over a range of seed sizes. There were also distinct differences between the LMWC profiles of immature seed, immature seed coat, and dry mature seed.

Across all entries, the immature cotyledon and immature seed coat consistently contained the highest GFS and the lowest RFOs concentrations while the dry seed had the lowest GFS and highest RFO content. Over most of the entries, the large seed size class of immature cotyledons contained higher GFS concentrations than either the medium or small seed size classes. Our investigation also revealed that the immature cotyledons exhibited the widest range in GFS content, emphasizing the strong potential for selecting sweeter genotypes for the fresh vegetable market. Unfortunately, we did not observe a strong enough relationship between GFS of immature and mature seeds to eliminate the need to analyze immature seeds directly.

Previous selection for larger faba bean seed, which extends the period of LMWC accumulation, has likely co-occurred with selection for increased LMWCs (Weber et al., 1996), although the specifics (intentional or unintentional selection) are up to speculation (Cleveland and Soleri, 2007). Developing inbred lines and/or mapping populations would help to further elucidate the genetic basis of carbohydrate metabolism of developing faba bean seeds.

224 Ultimately, selection of lines with specific quality characteristics such as high sucrose and/or low
225 RFO content will aid in improving the culinary acceptability and functionality of this species.

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Table 1. Mean, minimum, and maximum low molecular weight carbohydrates (% DW) for 40 faba bean populations separated into immature seed coat, immature cotyledon, and mature whole seed.

	Immature seed coat			Immature cotyledon+germ			Mature whole seed		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Glucose	2.15	0.5	5.53	0.94	0.17	3.67	*	*	*
Fructose	1.90	0.57	5.63	0.66	0.10	2.4	*	*	*
Sucrose	7.98	3.67	15.37	13.35	5.93	22.6	2.49	1.23	3.8
Raffinose	*	*	*	*	*	*	0.45	0.27	1.3
Stachyose	0.21	0	0.5	0.15	0	0.43	1.87	0.90	2.50
Verbascose	0.64	0.20	1.10	0.41	0.10	1.13	2.40	0.67	5.03

* Not consistently detectable

Figure 1. Total glucose, fructose, and sucrose (GFS) content (*se*±) of 40 faba bean populations separated into immature cotyledon and seed coat tissues. (n=3).

Figure 2. Total RFO (raffinose, stachyose, and verbascose) and sucrose content ($se\pm$) from mature seed of 40 faba bean populations. (n=3).

Figure 3. Scatter plot of immature cotyledon and seed coat total glucose, fructose, and sucrose (GFS) concentrations (% DW) from 40 faba bean populations by 100 seed weight. Regression lines are included. (n=3).

Figure 4. Scatter plot of total RFO (raffinose, stachyose, and verbascose) and sucrose concentrations (% DW) from mature seed of 40 faba bean populations by 100 seed weight. Regression lines are included. (n=3).