Simple Method of Isolation and Purification of α-Galactosides from Legumes

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A simple method for the isolation and purification of α-galactosides, raffinose family oligosaccharides (RFOs), from legumes has been developed. The method includes (i) imbibition of seeds, (ii) extraction with 50% ethanol, (iii) precipitation of RFOs, (iv) purification of RFOs on diatomaceous earth and charcoal, and (v) cation-exchange chromatography. The described method allows one to obtain high purity RFO preparations (90% for lentil and 80% for pea seeds, determined by HPLC-RI analysis) in the form of white, fine powder. Yields of α-galactosides isolated from 100 g of seeds of lentil and pea were 5.6 and 4.3 g, respectively.

Keywords: *α-Galactosides; raffinose family oligosaccharides (RFOs); lentil; pea*

INTRODUCTION

α-Galactosides, called also raffinose family oligosaccharides (RFOs), belong to low molecular weight, nonreducing sugars, soluble in water (Arentoft and Sorensen, 1992). They are α-(1→6)-galactosides linked to carbonc-6 of the glucose moiety of sucrose. Raffinose family oligosaccharides are widely distributed in the plant kingdom. Raffinose (trimer) is a representative of this group. Apart from raffinose, this group also includes stachyose (tetramer), verbascose (pentamer), ajugose (hexamer), and unnamed so far longer-chain oligosaccharides up to nonasaccharide (Cerning-Beroard and Filiatre-Verel, 1976, 1980). Large amounts of RFOs occur in the generative parts of higher plants where they perform protective physiological functions (Dey, 1985; Kuo et al., 1988; Larsson et al., 1993; Bachmann et al., 1994; Horbowicz and Obendorf, 1994).

However, from the nutritional point of view, RFOs are considered arduous factors because they are not hydrolyzed by mucosal enzymes in the small intestine of monogastric animals but pass into the lower gut where they are fermented with the deliberation of gas (Cristofaro et al., 1974; Saini and Gladstones, 1986; Prince et al., 1988). On the other hand, their ingestion in the form of pure compounds in diet increases the bifidobacteria population in the colon, which in turn contributes to human health in many ways (Minami et al., 1983; Tomomatsu, 1994).

These low-molecular weight oligosaccharides are of interest to many nutritionists as health food ingredients. Oligosaccharides are presently one of the most popular functional foods in Japan and they are added to such products as soft drinks, cookies, cereals, and candies (Tomomatsu, 1994). The RFOs can be obtained by extraction from plants, mainly from legume seeds, via enzymatic synthesis in vitro in the presence of glycosyltransferases, or via synthesis by the saccharide metabolic pathway in vivo (Muzquiz et al., 1999; Ichikawa et al., 1994).

In this work we propose a rapid method to obtain high purity RFO powder from lentil and pea seeds. This method, in view of its simplicity and high repeatability, may become of interest to the food industry.

EXPERIMENTAL PROCEDURES

Samples and Chemicals. Seeds of pea (*Pisum sativum* L. cv. Kastor) were supplied by Dr. Stanisław Stawiński from the Plant Breeding and Acclimatization Station at Przebędowo near Poznań (Poland). Seeds of lentil (*Lens culinaris* L.) were purchased from a local market (Spain). Diatomaceous earth, charcoal, and naphthoresorcinol were purchased from Sigma, Germany. Dowex 50WX8 100−200 Mesh was purchased from Serva, Switzerland. Ethanol, 2-propanol, and acetic acid were supported by POCh, Gliwice, Poland. Acetonitrile (HPLC grade) was purchased from ACROS-ORGANIC Belgium. Silica gel 60 F_{254} TLC plates, raffinose, and stachyose were from Merck, Darmstadt, Germany. Millipore FH (0.45 μm) membranes were supported by Millipore.

Determination of α-Galactoside Content in Seeds. Determination of soluble sugar (RFOs, sucrose, and others) content in seeds was carried out according to a procedure described previously by Muzquiz et al., 1999.

Isolation and Purification of RFOs. The general scheme of the isolation and purification procedure for RFOs from legume seeds is presented in Figure 1.

Imbibition of Seeds. First of all, legume seeds are imbibed in a determined volume of distilled water (required for full imbibition). The quantity of water used in this step depends on seed species and ranges from 80 to 120 mL/100 g of legume seeds. The imbibition of seeds was carried out at + 4 °C for 10−12 h. During this process the vessel with seeds should be shaken from time to time.

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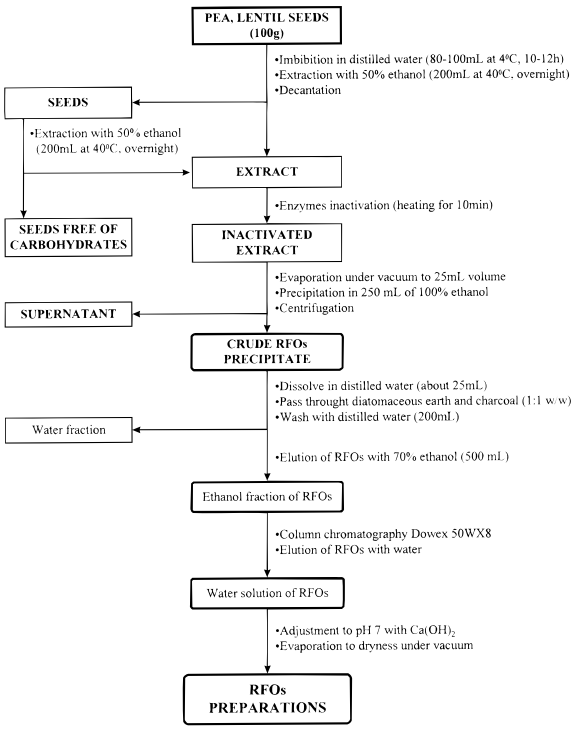

Figure 1. General scheme of isolation and purification of RFOs from pea and lentil seeds.

Extraction of RFOs. The imbibed seeds were then extracted with 200 mL of 50% ethanol (v/v) per 100 g of seeds at 40 °C overnight. The water that was not absorbed by seeds was used for preparation of proper ethanol concentration for extraction. After extraction the supernatant was decanted. The seeds were reextracted with fresh alcohol under the same conditions. The supernatants from two cycles of extraction were boiled under reflux for 10 min and combined together. In the case of lentil seeds extraction the supernatants containing any precipitate should be centrifuged before the next step.

RFOs Precipitation. The clear supernatant was concentrated on a rotary vacuum evaporator at 50 °C to the volume of 25 mL, placed in glass separator and dropped into 100% ethanol with continuous stirring. The ratio of water extract volume to volume of 100% ethanol should be 1:10. The crude RFOs precipitate was separated from supernatant by centrifugation at 3000 rpm for 15 min. The RFO precipitate was then placed into a vacuum desiccator in order to remove of any ethanol residue.

Purification of RFOs on Diatomaceous Earth and Charcoal. The crude RFO extract was dissolved in distilled water (25 mL) and placed onto diatomaceous earth and charcoal (1:1 w/w) located in a sintered glass funnel (pore size G4, 7 cm × 5 cm i.d.) and connected to a vacuum. The funnel was then washed with 200 mL of distilled water. The RFOs were eluted with 70% ethanol (500 mL). The presence of RFOs in the eluate was checked by reaction with naphthoresorcinol. Afterward, RFO alcohol solutions were concentrated to dryness on a rotary vacuum at 50 °C.

Cation-Exchange Chromatography. The purified RFOs (about 3 g) were dissolved in 10 mL of distilled water and then applied onto a Dowex 50WX8 column (12 × 1.5 cm i.d.) and washed with distilled water (50 mL) until oligosaccharides were not identified in the eluate. The presence of RFOs was monitored on TLC by reaction with naphthoresorcinol. The acidic solution of RFOs (pH 1.5) was adjusted to pH 7.0 using 4% freshly prepared $Ca(OH)_2$. The solution was then boiled for 2 min and centrifuged. Supernatant containing a high purity of RFOs was than evaporated to dryness on a rotary vacuum evaporator at 50 °C.

Qualitative Evaluation of RFO Composition Using Thin-Layer Chromatography (TLC). Qualitative evaluation of chemical composition of RFOs at each particular stage of purification was done by TLC according to Stahl, 1969, and Dey, 1990. For the separation of RFOs, 2-propanol−acetic acid−water (5:2:3 v/v) as mobile phase was used. The RFOs were visualized by naphthorezorcinol. For qualitative and quantitative separation of RFOs, silica gel 60 F_{254} TLC plates were used.

Determination of Total Soluble Sugars and α-Galactoside Content at Particular Stages of the Procedure. To 0.4 mL of an aqueous solution from each particular stage of purification, containing 2.0−15.0 μg of soluble sugars, 10 μL of 80% phenol in water (w/w) and 1 mL of concentrated H_2SO_4 were added. After mixing, the solution was kept at room temperature for 10 min and then cooled in a bath of cold water for 20 min. The same procedure was also performed for the standards. Resulting absorbance obtained using a DU-62 spectrophotometer (Beckman) at 485 nm was referred to the standard curve obtained for glucose (Fry, 1994).

HPLC Analysis of RFOs. The analysis of separation and quantification of RFOs from legume extracts was carried out by high performance liquid chromatography using a refraction index detector (HPLC-RI) (Frias et al., 1994). The analysis was performed on a HPLC chromatograph (Waters Associates, Milford, CT) equipped with a Waters model 510 pump, a Rheodyne model 7000 sample injector, a reflection type differential refractometr detector model R410 (Waters). The chromatographic system was controlled by an PC with a Maxima HPLC system controller software (Waters). A precolumn (0.32 cm i.d. × 4.0 cm) packed with C18 Porasil B and a m-Bondapak/carbohydrate column (0.39 i.d. × 30 cm) (Waters) were employed. Acetonitrile−distilled water (75:25 v/v, HPLC grade) was used as the mobile phase at the flow rate of 2.0 mL/min. Solvents were filtered through a Millipore FH (0.45 μm) membrane and degassed under helium. Injection volumes were 100 μL.

Standard Solution Preparation. Different amounts of raffinose and stachyose were dissolved in distilled water. Acetonitrile was added to each solution to obtain a composition similar to that of the mobile phase (75:25 v/v).

Legume Extract Preparation and Quantification. Different amounts of pea and lentil extracts were dissolved in distilled water. Acetonitrile was added to each solution to obtain a composition similar to that of the mobile phase (75:25 v/v). Both the legume extracts and standard solutions were filtered through a Millipore FH (0.45 μm) membrane before injection. Quantification of each sugar was accomplished by comparing the peak areas of the samples with those of the standard solutions. Commercial ciceritol and verbascose standards were not available, therefore ciceritol (trisaccharide) and verbascose (pentasaccharide) were identified based on standards obtained in our laboratories according to the previous work (Amarowicz et al., 1992; Bernabe et al., 1993; Frias et al., 1994) and by their retention times and were quantified using raffinose (trisaccharide) and stachyose (tetrasaccharide) as standards. A standard curve was plotted for each sugar and adjusted by using a method of least squares. The regression coefficients of the curves for raffinose and stachyose were always greater than 0.990.

RESULTS AND DISCUSSION

Analyzed legume seeds (pea and lentil) contained different amounts of total soluble sugars. The pea and lentil seeds differed from each other with percentage content of sucrose and RFOs in total soluble sugars (Table 1).

While pea seeds contain almost the same amounts of sucrose and RFOs (50% of the total soluble sugars), lentil seeds presented larger amounts of RFOs (77% of the total soluble sugars). During extraction with 50% ethanol, 83% and 87% of total soluble sugars from pea and lentil seeds, respectively, were eluted by alcohol, and 13−17% of carbohydrates remained uneluted. We

Table 1. Total Soluble Carbohydrates, Sucrose, and α-Galactoside Content in Pea and Lentil (in mg per g of seeds) Determined According to Muzquiz et al. (1999)

	pea	lentil
total soluble carbohydrates	107.3 (100%)	91.3 (100%)
sucrose	53.7 (50.2%)	30.1 (33.0%)
α-galactosides[a]	53.6 (49.8%)	61.2 (77.0%)

[a] Content of α-galactosides was calculated as a difference of total soluble sugars and sucrose.

Table 2. Percentage Content of α-Galactosides in RFO Preparations at Particular Stages of Purification Determined Spectrophotometrically and Recovery in Grams Obtained from 100 g of Seeds

stage	pea	lentil
extract	24.2	32.3
ethanol precipitate	59.1	57.5
diatomaceous earth and charcoal	76.0	72.2
Dowex 50WX8 chromatography	91.4	83.2
recovery	4.3	5.6

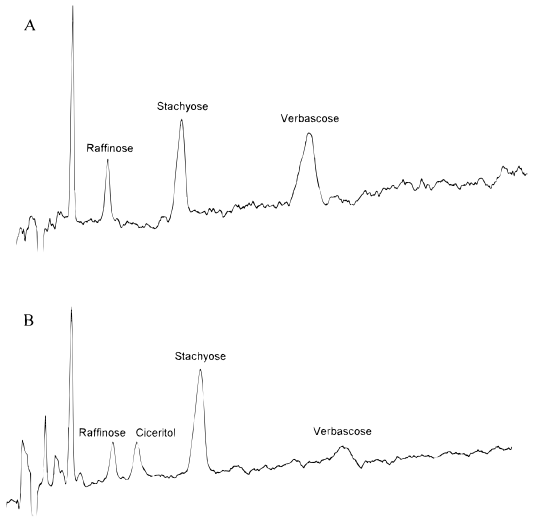

Figure 2. Chromatograms of RFO preparations: (A) pea; (B) lentil.

found that prolongation of extraction time, higher temperature, and concentration of ethanol had no essential influence on the yield of the extraction process (data not presented).

Table 2 shows percentage content of oligosaccharides in RFO preparations at particular stages of purification, where the content of sugars was determined spectrophotometrically. These results show that at each stage of purification the content of RFO increased. Finally, RFOs preparations from pea and lentil seeds were characterized by high purity: 91.4 and 83.2%, respectively.

Figure 2 shows chromatograms of RFO preparations from pea and lentil. The quantification of different sugars of the raffinose family in RFO preparations is presented in Table 3. Stachyose was the dominant oligosacharide in lentil seeds that also presented a large amount of ciceritol. Peas, however, did not contain ciceritol and presented a large amount of verbascose. The raffinose level was 7.2% and 5.6% in pea and lentil, respectively. The content of sucrose in pea and lentil RFO preparations was similar and reached ca. 12%.

In the procedure of α-galactosides isolation and purification from legume seeds, five main stages can be distinguished: (i) imbibition of seeds; (ii) extraction of RFOs; (iii) RFO precipitation; (iv) purification of RFOs

Table 3. Average Percentage Content of Particular α-Galactosides, Sucrose, and Ciceritol in RFO Preparations Obtained from Pea and Lentil Seeds

sugars	pea	lentil
sucrose	11.48	12.15
raffinose	7.23	5.63
ciceritol	0.00	9.26
stachyose	29.28	39.54
verbascose	40.28	11.51
total	88.27	78.32

on diatomaceous earth and charcoal; (v) cation-exchange chromatography.

In view of the compact structure of legume seeds the extraction process of RFOs is preceded by the imbibition step, which causes the loose of seed structure and facilitates penetration of solvents. Imbibition performed in a predeterminated volume of distilled water and at +4 °C reduces the elution of albumins soluble in water and the activity of enzymes, mainly galactosidases. Many authors recommend extraction of RFOs from flour by 80% ethanol or methanol at boiling temperature in order to reduce enzymatic activity (Vidal-Valverde et al., 1993; Kwasnicka et al., 1996; Johansen et al., 1996; Frias et al., 1996). We stated that conditions of RFO extraction described in our work (whole seeds, 50% ethanol, 40 °C) fully protect oligosaccharides against galactosidase degradation and allow for more selective extraction (data not presented). Unfortunately, extraction from whole seeds seems to require a longer extraction time. In choosing the extraction method, different factors should be taken into consideration, like aim, scale, costs, and others.

Furthermore, imbibition and ethanol extraction of whole legume seeds give three essential advantages: (i) the seed coat performs the function of a sieve and prevents elution of high molecular weight compounds; (ii) it is easy to operate (for example, separation of extract from seeds does not require centrifugation and other labor-consuming treatments) (Gulewicz, 1988); (iii) the remained residue in the form of extracted whole seeds can be a convenient source of natural products for further studies and industrial application.

During the first stage of purification of RFOs (precipitation with 100% ethanol), a significant amount of sucrose and monosaccharides remain in the supernatant. Unfortunately, crude RFO preparations after this step still have the form of a dark brown oil. Significant purification of crude oligosaccharides was obtained after percolation of a water solution of RFOs through diatomaceous earth and charcoal. After this step, the RFO preparations have the form of a white powder but still contain impurities of the basic compounds such as amino acids and others. Efficient removal of these compounds from RFO preparations was achieved after application of cation-exchange chromatography on a Dowex 50WX8 column.

RFO preparations from pea and lentil seeds contained 88% and 78% soluble sugars, respectively. The amount of α-galactosides isolated from 100 g of seeds was 4.3 g for pea and 5.6 g for lentil (Table 2). These amounts constituted 40.1% and 61.0%, respectively, of total soluble sugars in the studied plants. These results seems fully satisfactory in the light of incomplete extraction (ca. 85%) and the fact that soluble sugars from pea and lentil seeds contain considerable amounts of sucrose, ca. 50 and 33%, respectively (Kuo et al., 1988; Frias et al., 1996).

The described method may appear very useful upon investigation of the biological activity of raffinose family oligosaccharides. It allows one to obtain highly purified RFO preparations mainly from pea and lentil seeds in a relatively short time. The byproducts of this method are seeds containing a high amount of protein and extracts containing many natural products, which may attract attention for industrial uses.

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