JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Improved Method To Obtain Pure α-Galactosides from Lupin Seeds

CRISTINA MARTÍNEZ-VILLALUENGA,† JUANA FRIAS,† KRZYSZTOF GULEWICZ,§ AND CONCEPCIÓN VIDAL-VALVERDE*,†

Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, Madrid 28006, Spain, and Institute of Bioorganic Chemistry (PAS), Noskowskiego 12/14, Poznan 61-704, Poland

Improvement of a previously described method of purification of α-galactosides, members of the raffinose family of oligosaccharides (RFOs), has been developed for lupins. The considerable amount of sucrose present in the RFO preparations obtained by the previous method has been removed by modifying the purification stage on diatomaceous earth and charcoal. The present method allows for the preparation of high-purity RFOs containing ∼99.4% of RFOs in the form of a white fine powder, which provides new perspectives for the production of pure α-galactoside preparations for their use as prebiotics in functional foods.

KEYWORDS: α-Galactosides; raffinose family oligosaccharides (RFOs); lupins

INTRODUCTION

α-Galactosides, called also raffinose family oligosaccharides (RFOs), are widely distributed in the plant kingdom. Large amounts of RFOs occur in the generative parts of higher plants, where they perform protective physiological functions (*1*−*4*). In legumes, RFOs accumulate during seed development; raffinose and stachyose are formed de novo during seed maturation (*5*, *6*) and are carbon reserves utilized during germination (*7*, *8*). Legume seeds have a high α-galactoside content, and their consumption has been associated with the production of flatulence, because these oligosaccharides are not hydrolyzed in the small intestine, due to the absence of α-galactosidases in monogastric animals, and they are fermented in the lower intestine (*9*, *10*). This problem has been considered to be the single most important factor that deters people from eating this nutritious food (*10*).

It has been considered recently that pure RFOs are prebiotics. RFOs promote the growth of the bifidobacteria population (*11*, *12*) and, consequently, there is a great deal of interest in using prebiotic oligosaccharides as ingredients in functional foods to manipulate the composition of colonic microflora, contributing to human health in many ways.

There are many prebiotic oligosaccharides known, principally in the Japanese market, and they are added as ingredients in a large number of products such as soft drinks, cookies, cereals, and candies (*12*). α-Galactosides from soybean are the only legume oligosaccharides on the market, and the main producer is Japan (*13*). In the United States and Europe RFOs are also available on the market; however, the market leaders are fructo-oligosaccharides in these regions.

* Author to whom correspondence should be addresse d(telephone +34 915622900, ext. 241; fax +34 915644873; e-mail ificv12@ifi.csic.es).
† Instituto de Fermentaciones Industriales (CSIC).
§ Institute of Bioorganic Chemistry (PAS).

These low molecular weight oligosaccharides are present in different seeds, but lupins are one of the legumes with the most of α-galactoside content (7−15%) (*8*, *14*−*17*). The sweet lupins, containing very low levels of alkaloids, can be a good source of RFOs. In 2000 a paper was published describing a simple method for the isolation and purification of α-galactosides from lentils and peas (*18*), but the obtained RFO preparations contained sucrose in considerable amounts (11 and 12%, respectively). In the present work, a modification of the previously published method is presented that allows lupin RFOs free of sucrose to be obtained. It creates new perspectives for the production pure α-galactoside preparations for their use as prebiotics in functional foods.

MATERIALS AND METHODS

Samples and Chemicals. Sweet lupin seeds of *Lupinus albus* L. cv. Multolupa were obtained from the Agricultural Research and Technology Development Service of the Agriculture and Commerce Council of the Junta de Extremadura (Spain). Seeds were cleaned and stored in a polyethylene container at 4 °C until used. Diatomaceous earth, charcoal, and naphthoresorcinol were purchased from Sigma, Darmstadt, Germany. Dowex 50WX8, 100−200 mesh, was purchased from BDH Laboratories (Poole, U.K.). Ethanol, 2-propanol, and acetic acid were supplied by Scharlau (Dusseldorf, Germany). Acetonitrile (HPLC grade) was purchased from Acros−Organic (Geel, Belgium). Silica gel 60 F_{254} TLC plates, sucrose, raffinose, and stachyose were obtained from Merck, Darmstadt, Germany. Millipore FH (0.45 μm) membranes were obtained from Millipore (Bedford, MA).

Isolation of RFOs. Lupin seeds were submitted to different selective extractions according to a procedure previously described by Gulewicz et al. (*18*) (**Figure 1**).

Purification of RFOs. The purification scheme of RFOs is shown in **Figure 1**. RFO precipitate was dissolved in 25 mL of distilled water and suspended in a glass funnel (pore size G4, 7 cm × 5 cm i.d.) with diatomaceous earth and charcoal (1:1 w/w) and connected to vacuum.

10.1021/jf040131+ CCC: $27.50
Published on Web 10/16/2004

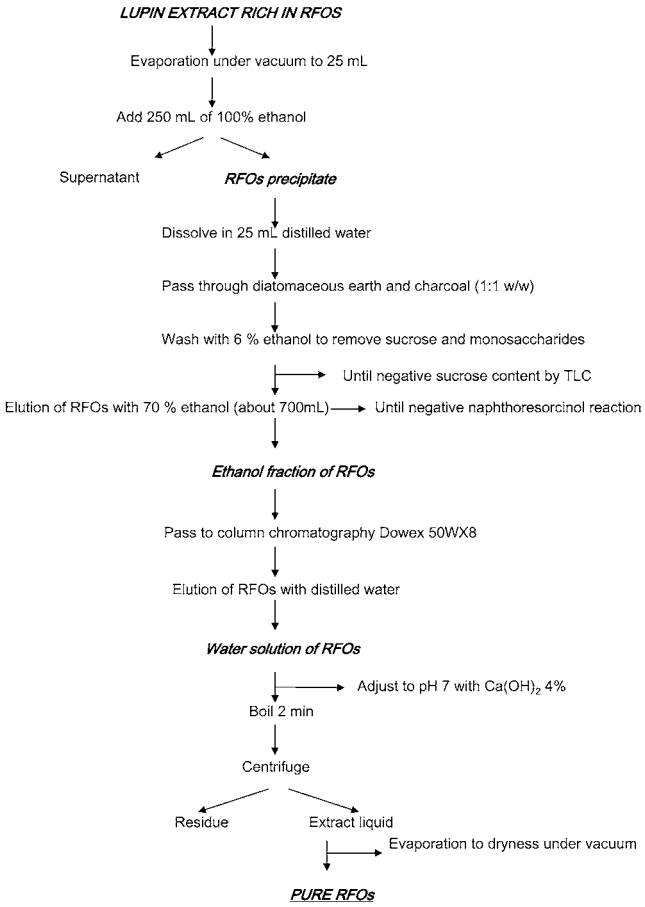

Figure 1. Purification of lupin extract rich in RFOs.

The funnel was washed with 6% ethanol to remove sucrose. The absence of sucrose in the eluate was confirmed by thin-layer chromatography (TLC) according to the method of Dey (*7*). TLC of carbohydrates was performed on silica gel 60 F_{254} plates with 2-propanol/acetic acid/water (5:2:3 v/v). Carbohydrates were visualized with naphthoresorcinol. After the absence of sucrose was confirmed, RFOs were eluted with 70% ethanol until negative naphthoresorcinol reaction. The ethanol RFO fraction was concentrated to dryness on a vacuum evaporator at 50 °C. The RFOs were dissolved in 10 mL of distilled water, transferred into a Dowex 50WX8 column (12 × 1.5 cm i.d.), and washed with distilled water (50 mL) until negative naphthoresorcinol reaction. The acid solution of RFOs (pH 2) was adjusted to pH 7 with 4% freshly prepared $Ca(OH)_2$. The solution was then boiled for 2 min and centrifuged for 10 min at 10000 rpm. Supernatant containing a high-purity RFO was then evaporated to dryness on a vacuum evaporator at 50 °C.

Determination of Total Soluble Carbohydrate Content. Sucrose and α-galactoside contents were determined by HPLC following the procedure described by Granito et al. (*19*).

RESULTS AND DISCUSSION

Figure 2 shows chromatograms of RFO preparations from lupins obtained as previously described by Gulewicz et al. (*18*) (method A) and via the improved method described in this work (method B). It can be observed that the chromatographic peak of sucrose was sharply reduced using method B, whereas those of raffinose, stachyose, and verbascose increased. These peaks were quantified, and results are shown in **Table 1**. The improved method described in this paper increased to 99.4% the purity of RFO preparations, compared with 75.1% obtained using method A. The RFO powder obtained presented 12.6% raffinose, 62.3% stachyose, 24.5% verbascose, and only a trace amount of sucrose (0.8%) (**Table 1**).

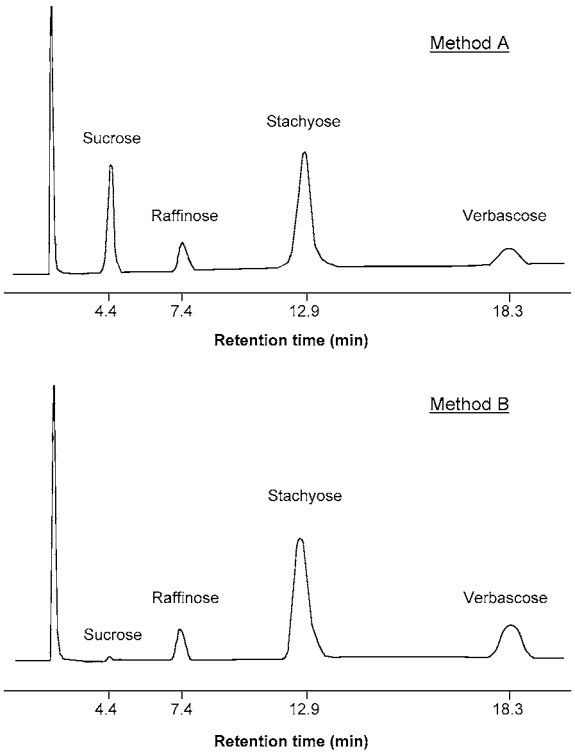

Figure 2. HPLC chromatograms of RFO preparations.

Table 1. Percentage of α-Galactosides and Sucrose in the RFO Preparations from Lupin Obtained by Methods A and B

sugar	method A[a]	method B[b]
sucrose	20.22 ± 0.11	0.82 ± 0.01
raffinose	9.65 ± 0.33	12.56 ± 0.91
stachyose	52.99 ± 2.13	62.33 ± 0.48
verbascose	12.50 ± 1.01	24.47 ± 0.05
total α-galactosides	**75.14** ± 2.86	**99.36** ± 1.43

[a] Method A: according to that of Gulewicz et al. (*18*). [b] Method B: present improved method.

There are five stages in the improved method of isolation and purification of α-galactosides from legume: (i) imbibition of seeds, (ii) extraction of RFOs, (iii) RFO precipitation, (iv) purification of RFOs on diatomaceous earth and charcoal, and (v) cation-exchange chromatography. In the purification stage with diatomaceous earth and charcoal (iv), percolation of RFOs with water used in the method described by Gulewicz et al. (*18*) was not efficient for the total removal of monosaccharides, sucrose, and other impurities. Using the 6% ethanol solution makes possible entire elution of sucrose, which has been confirmed by HPLC analysis. Finally, using a higher concentration of ethanol (70%) led to the total elution of RFOs, and the obtained preparation shows very high purity (>99%).

The removal of RFOs from legume seeds has been widely studied from the nutritional point of view, in order to obtain legume flour with low flatulence-causing compounds (*20*−*22*), but little attention has been paid to the extraction and purification of these α-galactosides as prebiotic substances. Lupin seeds have been previously used as a source of RFOs, but in those studies the purity of the isolated has not been reported (*15*). Kim et al. (*23*) optimized the extraction of oligosaccharides from soybean meal with either water at 50 °C or 10% ethanol, and purification was carried out by ultrafiltration. These authors found fructose, sucrose, raffinose, and stachyose in their concentrates. Gulewicz et al. (*18*) obtained soluble sugar preparations containing 11.5%

sucrose, 7.2% raffinose, 29.3% stachyose, and 40.3% verbascose from peas and 12.2% sucrose, 5.6% raffinose, 9.3% ciceritol, 39.5% stachyose, and 11.5% verbascose from lentils. The improved method described in this paper offers a simple and rapid procedure for obtaining high-purity RFO preparations free of monosaccharides and disaccharides from lupin seeds, and it could be applied to other legumes. The pure RFOs can be offered as functional food ingredients like prebiotics, which can contribute to human health.

LITERATURE CITED

(1) Dey, P. M. D-Galactose containing oligosaccharides. In *Biochemistry of Storage Carbohydrates in Green Plants*; Dey, P. M., Ed.; Academic Press: London, U.K., 1985; pp 5−129.

(2) Kuo, T. M.; Van Middlesworth, F. K.; Wolf, W. J. Content of raffinose oligosaccharides and sucrose in various plant seeds. *J. Agric. Food Chem*. **1988**, *36*, 32−36.

(3) Larsson, S.; Johansson, L. A.; Svenningsson, M. Soluble sugars and membrane lipids in winter wheats (*Triticum aestivum* L.) during cold acclimation. *Eur. J. Agron.* **1993**, *1*, 85−90.

(4) Horbowicz, M.; Obendorf, R. L. Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. *Seed Sci. Res.* **1994**, *4*, 385−405.

(5) Lowell, C. A.; Kuo, T. M. Oligosaccharide metabolism and accumulation in developing soybean seeds. *Crop Sci.* **1989**, *29*, 459−465.

(6) Frias, J.; Vidal-Valverde, C.; Kozlowska, H.; Gorecki, R.; Honke, J.; Hedley, C. L. Evolution of soluble carbohydrates during the development of pea, faba bean and lupin seeds. *Z. Lebensm. Unters. Forsch.* **1996**, *203*, 27−32.

(7) Dey, P. M. Oligosaccharides. In *Methods in Plant Biochemistry*; Dey, P. M., Ed.; Academic Press: New York, 1990; Vol. 2, pp 189−218.

(8) Frias, J.; Diaz-Pollan, C.; Hedley, C. L.; Vidal-Valverde, C. Evolution and kinetics of monosaccharide, disaccharide and α-galactosides during germination of lentils. *Z. Lebensm. Unters. Forsch*. **1996**, *202*, 35−39.

(9) Cristofaro, E.; Mottli, F.; Whurmann, J. J. Involvement of raffinose family oligosaccharides in flatulence. *Sugars in Nutrition*; Sepple, H. I., McNut, K. W., Eds.; Academic Press: New York, 1974; pp 313−363.

(10) Price, K. R.; Lewis, J.; Wyatt, G. M.; Fenwick, R. G. Flatulence—causes, relation to diet and remedies. *Nahrung* **1988**, *32*, 609−626.

(11) Gibson, G. R.; Roberfroid, M. D. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* **1995**, *125*, 1401−1412.

(12) Tomomatsu, H. Health effects of oligosaccharides. *Food Technol.* **1994**, *48*, 61−65.

(13) Crittenden, R. G.; Playne, M. J. Production, properties and applications of food-grade oligosaccharides. *Trends Food Sci. Technol.* **1996**, *7*, 353−361.

(14) Van Kempen, R. J.; Hughes, R. J. The nutritive value of lupin for pigs and poultry. *Proccedings of the First Australian Lupin Technical Symposium*; 1994; pp 49−57.

(15) Muzquiz, M.; Burbano, C.; Pedrosa, M. M.; Folkman, W. Y.; Gulewicz, K. Lupin as a potential source of raffinose family oligosaccharides. Preparative method for their isolation and purification. *Ind. Crops Prod.* **1999**, *9*, 183−188.

(16) Ruiz-López, M. A.; García-López, P. M.; Castañeda-Vazquez, H.; Zamora, N. J. F.; Garzón-De la Mora, P.; Bañuelos Pineda, J.; Burbano, C.; Pedrosa, M. M.; Cuadrado, C.; Muzquiz, M. Chemical composition and antinutrient content of three *Lupinus* species from Jalisco, Mexico. *J. Food Compos. Anal*. **2000**, *13*, 193−199.

(17) Saini, H. S.; Gladstones J. S. Variability in the total and component galactosyl sucrose oligosaccharides of *Lupinus* species. *Aust. J. Agric. Res.* **1986**, *37*, 157−166.

(18) Gulewicz, P.; Ciesiolka, D.; Frías, J.; Vidal-Valverde, C.; Frejnagel, S.; Trojanowska, K.; Gulewicz, K. Simple method of isolation and purification of α-galactosides from legumes. *J. Agric. Food Chem.* **2000**, *48*, 3120−3123.

(19) Granito, M.; Frías, J.; Doblado, R.; Guerra, M.; Champ, M.; Vidal-Valverde, C. Nutritional improvement of beans (*Phaseolus vulgaris*) by natural fermentation. *Eur. Food Res. Technol.* **2002**, *214*, 226−231.

(20) Frias, J.; Vidal-Valverde, C.; Sotomayor, C.; Díaz-Pollan, C.; Urbano, G. Influence of processing on available carbohydrate content and antinutritional factors of chickpeas. *Eur. Food Res. Technol*. **2000**, *210*, 340−345.

(21) Sanz, M. A.; Blazquez, I.; Sierra, I.; Medrano, M. A.; Frias, J.; Vidal-Valverde, C.; Hernandez, A. Nutritional evaluation of ethanol extracted lentil flours. *J. Agric. Food Chem.* **2001**, *49*, 1854−1860.

(22) Vidal-Valverde, C.; Sierra, I.; Frias, J.; Prodanov, M.; Sotomayor, C.; Hedley, C. L.; Urbano, G. Nutritional evaluation of lentil flours obtained after short time soaking processes. *Eur. Food Res. Technol*. **2002**, *215*, 138−144.

(23) Kim, S.; Kim, W.; Hwang, K. Optimization of the extraction and purification of oligosaccharides from defatted soybean meal. *Int. J. Food Sci. Technol*. **2003**, *38*, 337−342.

Received for review March 15, 2004. Revised manuscript received August 5, 2004. Accepted August 19, 2004. This work is a result of a bilateral scientific cooperation joint project between Poland (PAS) and Spain (CSIC); it has been supported by CICYT AGL-2001-2302, and it is part of C.M.-V.'s thesis.

JF040131+