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Araştırma/*Research*

CONCENTRATION OF D-PINITOL in CAROB EXTRACT by USING MULTI-STAGE ENRICHMENT PROCESSES

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Abstract

D-pinitol, a cyclic sugar alcohol, is claimed to be a potential therapeutic compound related to the illnesses arising from insulin mechanism. Carob is a rich source of this compound and has recently begun to be used in different separation and purification studies for obtaining D-pinitol. In this study, different enrichment processes were applied to concentrate the D-pinitol content of the carob extract. To determine the effectiveness of the processes applied for concentration of the target compound in carob extract, removal of other impurities (mainly sugars) was used as an important indicator. According to the results; the highest increase in D-pinitol concentration was observed in the enrichment process combining the techniques such as ethanol fermentation, membrane filtration and solvent extraction. At the end of this multi-stage process, D-pinitol concentration increased approximately four-fold (37.53 g/100 mL dry weight) when compared to its initial level (9.38 g/100 mL dry weight) in carob extract.

Keywords: Carob, D-pinitol, enrichment, fermentation, ultrafiltration

KEÇİBOYNUZU EKSTRAKTINDA BULUNAN D-PİNİTOLÜN ÇOK AŞAMALI ZENGİNLEŞTİRME PROSESİ İLE KONSANTRASYONU

Özet

D-pinitol insülin mekanizması ile ilişkili hastalıkların tedavisinde kullanılabilme potansiyeli olan bir şeker alkolüdür. Bu şeker alkolü bakımından zengin olan keçiboynuzu meyvesi, D-pinitolün ayrıştırılması ve saflaştırılması amacıyla son yıllarda gerçekleştirilen birçok araştırmada materyal olarak kullanılmıştır. Bu çalışmada keçiboynuzu ekstraktının D-pinitol içeriğini konsantre etmek amacıyla farklı zenginleştirme prosesleri kullanılmıştır. Hedef bileşiğin konsantre hale getirilmesi amacıyla kullanılan tekniklerin etkinliğini belirlemede keçiboynuzu ekstraktında bulunan diğer safsızlıkların (esas olarak şekerlerin) uzaklaştırılması önemli bir gösterge olarak kullanılmıştır. Elde edilen bulgulara göre; D-pinitol konsantrasyonundaki maksimum artış etanol fermantasyonu, membran filtrasyonu ve solvent ekstraktsiyonu gibi tekniklerin beraber uygulanmasıyla elde edilmiştir. Bu çok aşamalı prosesin sonunda, keçiboynuzu ekstraktının sahip olduğu başlangıç D-pinitol miktarı (9.38 g/100 mL kuru madde) yaklaşık dört kat konsantre (37.53 g/100 mL kuru madde) hale getirilmiştir.

Anahtar Kelimeler: Keçiboynuzu, D-pinitol, zenginleştirme, fermantasyon, ultrafiltrasyon

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INTRODUCTION

Carob is remarkably rich in minerals and carbohydrates but it also contains a large amount of condensed tannins (1, 2). In terms of carbohydrate composition, carob is particularly rich in saccharose (29.9-38.4 g/100g dry weight-DW), glucose (3.3-3.72 g/100g DW) and fructose (5.58-11.5 g/100g DW) (3, 4). Except for these sugars, xylose, maltose and raffinose have also been reported to be found in carob (5). Carob has also cyclic sugar alcohols-cyclitols (e.g. D-pinitol, *myo*-inositol, *chiro*-inositol, ononitol, sequoitol and bornesitol) as in the other plants (e.g. soybean) of Leguminosae family. Among these, D-pinitol concentration is very high (5-8 g/100 g DW) (6, 7).

D-pinitol, 3-O-methyl-D-*chiro*-inositol, is a water soluble, bioactive cyclitol and mainly found in the plants of Leguminosae family (8). D-pinitol, its derivatives and metabolites in nutritional and medicinal compositions have been reported to have possibility of being useful for lowering plasma free fatty acid levels and for treating conditions associated with insulin resistance, such as diabetes mellitus and its chronic complications; obesity; hypertension; cardiovascular disease; AIDS; cancer; malnutrition; aging; polycystic ovary syndrome etc. (9-13). It is not synthesized or transformed to the other compounds found in its metabolic pathway in living tissues (14). Therefore, its uptake should be provided by foods or food supplements.

Soybean has been mainly used as the main source for D-pinitol extraction. For this purpose, different multi-stage processes including most frequently the techniques such as microbial fermentation, chromatographic separation and solvent extraction have been carried out in that studies (15, 16). However, alternative sources containing higher levels of D-pinitol have begun to be searched because of containing only 1 % (DW) of D-pinitol in soybean. In this respect, carob with its high D-pinitol content (5-8 % DW) has taken much more attention in recently. But having more impurities (especially high sugar content – 40-50 % (DW) of carob) extracting of this functional compound from carob matrix is a major problem and the separation/purification can be obstructed from these compounds. Therefore, different investigations have been performed to separate sugars from cyclitols. For this purpose, especially anionic and cationic ion exchange resins have been used (17, 18).

The main aim of this study was to find an alternative process to the known techniques in extraction of D-pinitol from carob. For this purpose, the extract subjected to different processes was monitored for the variations of D-pinitol and other impurities (mainly in sugars of saccharose, glucose and fructose) in the carob extract.

MATERIAL and METHODS

Material

Broken and deseeded carob kibbles were purchased from a local factory in Antalya, Turkey at the end of summer season of 2013. The carob kibbles were put into plastic lid boxes and stored at 4 °C until processing and analyzing.

The Enrichment Processes

Different techniques such as clarification, ethanol fermentation, ultrafiltration (UF) and solvent extraction were combined to investigate the synergic effect of the techniques on removal of other impurities during concentration of D-pinitol from carob extract. Therefore, two different processes were designed and performed (Figure 1). These processes were similar in respect to the application of extraction, enzyme treatment and fermentation steps although having some differences (Figure 1 A and B).

The carob extraction conditions were 1:4 material-hot water ratio, 80 °C and 2 hours (19). After extraction, the carob extract was subjected to coarse filtration with cellulose filter. The clarification of the carob extract was performed in two steps: Enzyme treatment and hot clarification. In the enzyme treatment, both pectolytic and inversion activity were applied. The parameters used in clarification stage were determined with preliminary tests. For pectolytic enzyme treatment (50 °C, 50 µL/L (for each enzyme) and 45 minutes), a mixture of pectolytic enzymes (Pectinex Smash XXL and Pectinex Ultra Clear, Novozymes A/S, Denmark) was used. The main purposes of using the pectolytic enzyme mixture in both processes were to increase flocculation of pectic compounds thereby separation of them from the extract during the hot clarification stage and to transform the pectin found in the carob pod into an easily metabolizable form for the *Saccharomyces*

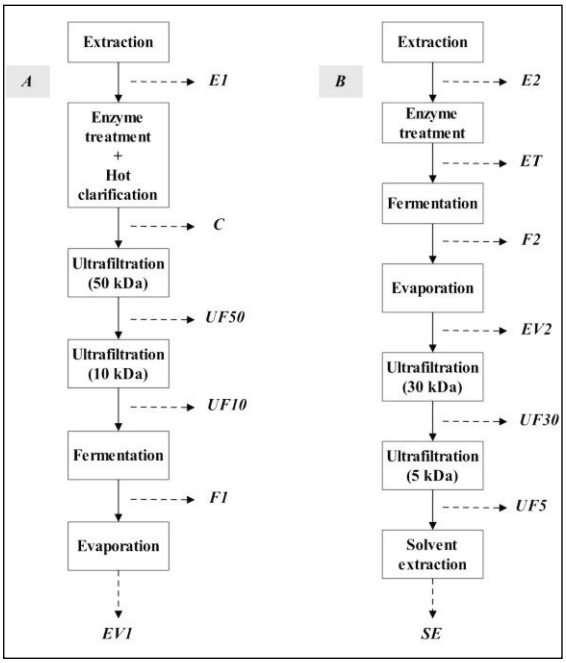

Figure 1. Schematic diagram of the processes and sample coding (A: First process, B: Second process)

cerevisiae yeast during the ethanol fermentation stage, respectively. The main monomers of pectin are glucose, L-arabinose and D-galacturonate. From these monomers, only glucose can be used by wild-type *S. cerevisiae* strains. However, significant progress about utilization of pentoses such as D-xylose and L-arabinose by metabolically engineered S. cerevisiae strains has been made recently (20, 21). For invertase treatment, invertase (Invertase, MP Biomedicals, California, USA) enzyme was used and the optimum conditions (30 °C, 1 % inoculation rate (v/v) and 2 hours) for invertase usage were determined with preliminary tests. Fining agents of bentonite (5 % w/v), gelatin (5 % w/v) and kieselsol (1.5 % v/v) were used in the hot clarification applied at 50 °C for 3 hours. The agents were added in different proportions (4-6 g/L for bentonite; 1-3 g/L for gelatin; 3-6 mL/L for kieselsol) to the carob extract heated to 50 °C and incubated in this temperature in a water bath (Jeio Tech, BS-06/31, Seoul, Korea). The optimum combination (6 g/L for bentonite; 3 g/L for gelatin; 4.5 mL/L for kieselsol) of fining agents was determined with preliminary tests. After the incubation, the clarified carob extract was obtained with coarse filtration.

Clarified carob extracts were subjected to UF process (2.5 bar for inlet, 0.5 bar for outlet pressure; room temperature, permeate:retentate ration: 80%, Sartorius Stedim, Sartocon Slice 200, Goettingen, Germany). Molecular weight cut-off (MWCO) rates of the UF membrane filters were different for the two processes (50 and 10 MWCO for the first and 30 and 5 MWCO for the second process).

The carob extract obtained from the second UF process as permeate was subjected to the ethanol fermentation. In the fermentation, *S. cerevisiae* (ATCC 36858) was chosen as the fermentation yeast because of its performance in sugar assimilation and conversion of D-pinitol derivatives to the free D-pinitol form (16). The ethanol fermentation conditions were performed according to Turhan, Bialka, Demirci and Karhan (19). The fermentation was carried out with a bio-reactor (Sartorius Stedim, Biostat B Plus, Germany) using the conditions of % 1 (v/v) inoculation rate, 30 °C, 150 rpm and 30 hours. The pH value of the extract was adjusted to 5.5 only in the beginning of the fermentation with a 4 N Na_2CO_3 solution and during the fermentation, no solution was added to stabilize the pH. For the fermentation, carob extract did not enrich with any additional nutrient. Therefore the yeast was obliged to use the nutrients inherently found in the extract. The incubation was terminated according to the time the yeast begun to use D-pinitol as the carbon source (30 hours). After the process, the fermented carob extract was evaporated in order to remove the ethanol from the final product (Evaporation with Heidolph, HEI-VAP Value, Germany at 40 °C, 200 mbar and 20 rpm).

The second enrichment process was slightly different from the first one. The process parameters of the second enrichment process for extraction, enzyme treatment, ethanol fermentation, evaporation and ultrafiltration were the same with the first process. Accordingly; the carob extract was only subjected to enzyme treatment but was not clarified, then fermented. After removal of ethanol by evaporation, the extract was gradually ultrafiltered (first with a 30 kDa MWCO then 5 kDa MWCO). Final enrichment process for the carob extract was solvent extraction with ethanol (Figure 1-B). This treatment was performed with reference to Shin, Jeon, Kim and Choi (16), but with slight modifications. Accordingly; the carob extract was first evaporated to 50 °Bx then mixed with 96 % of ethanol solution in the range of 1:3 (extract:ethanol).

The mixture was stirred with a vortex and centrifuged at 3000 rpm for 5 minutes. After centrifugation, supernatant was evaporated fractionally with distilled water to remove ethanol from the extract.

Analytical Methods

Determination of Sugars and D-pinitol

D-pinitol, saccharose, glucose and fructose compounds were determined according to the method described by Tetik, Turhan, Oziyci and Karhan (7) with a HPLC solvent delivery system (Shimadzu LC-20 AD) equipped a guard column (CARBOsep Coregel 87P, 4 X 20 mm^2; Transgenomic, Omaha, NE, USA) connected to an analytical column (CARBOsep Coregel 87P, 7.8 X 300 mm^2; Transgenomic, Omaha, NE,USA), a Shimadzu RID-10A refractive index detector, Shimadzu SIL-20A autosampler and a Shimadzu CTO-20A column oven. Chromatographic conditions were: 85 °C, 0.6 mL/min of flow rate for mobile phase (Milli-Q water), 20 µL injection volume at isocratic elusion. The samples diluted 1/200 times were passed through a 0.45 µm membrane filter (CHROMAFIL® PET-45/25; Macherey-Nagel, Düren, Germany) before injection. The external standards of D-pinitol, saccharose, glucose and fructose were (50-500 µg/mL) used for the quantification.

Determination of Soluble Solids and Total Dry Matter

The soluble solids and total dry matter were analyzed according to Cemeroğlu (22). The soluble solids measurements were carried out using an Abbe refractometer (ATAGO®, NAR-1T, Japan) at 25 °C. The total dry matter analysis was carried out using a universal oven at 70 °C for 48 hours (Memmert, UF 110 Plus, Germany). The total soluble solids values were used for the normalization of the D-pinitol concentrations obtained after from each process was applied and the total dry matter values were used to express the last D-pinitol concentrations reached on a basis of dry matter.

Determination of Ethanol

Ethanol concentrations of the fermented carob extracts were measured with an YSI 2700 bioanalytical system (YSI Life Sciences, OH, USA). An ethanol standard of 3.20 g/L was used for the calibration of the equipment. The samples were diluted according to the calibration range of the system before measurement.

Statistical Analysis

The data were assessed by using SAS (SAS Institute Inc., Cary, NC, USA). Factorial analysis of variance and Duncan's multiple comparison test (when necessary) were used at significance level, P=0.05. Values of all parameters were the average of four different measurements and expressed in the tables/graphs as mean ± standard deviation.

RESULTS and DISCUSSION

Variation in Sugar Contents of The Carob Extracts During The First and Second Processes

Sugars (approximately 40-50 % based on dry matter of the carob fruit) are the major obstructive impurities in carob extract for D-pinitol extraction.

Table 1. Sugar and D-pinitol contents of the samples from different stages of the first and second processes

Process	Stage	Saccharose (g/L)	Glucose (g/L)	Fructose (g/L)	D-pinitol (g/L)	Soluble Solids (°Bx)
First	E1	53.00±2.08	10.74±0.59	13.87±2.64	10.14^{b}±0.47	11.03^{a}±1.37
	C	n.d.	37.92±1.15	34.86 ±4.07	9.45^{bc}±0.52	11.38^{a}±0.61
	UF50	n.d.	39.58±2.37	34.04±2.54	9.74^{bc}±0.71	10.88^{a}±0.73
	UF10	n.d.	39.06±2.70	34.41±2.20	9.65^{bc}±1.14	11.05^{a}±0.70
	F1	n.d.	n.d.	n.d.	8.76^{c}±0.50	6.08^{b}±0.56
	EV1	n.d.	n.d.	n.d.	12.24^{a}±0.47	6.09^{b}±0.72
Second	E2	59.52±0.65	12.10±0.26	12.48±0.69	12.15^{e}±0.15	12.85^{c}±0.07
	ET	n.d.	46.18±0.11	40.24±0.21	13.24^{e}±0.13	13.30^{b}±0.00
	F2	n.d.	n.d.	n.d.	16.55^{d}±0.84	9.05^{e}±0.07
	EV2	n.d.	n.d.	n.d.	22.34^{bc}±0.08	9.85^{e}±0.07
	UF30	n.d.	n.d.	n.d.	22.71^{b}±0.44	11.40^{d}±0.14
	UF5	n.d.	n.d.	n.d.	21.34^{c}±0.41	8.60^{f}±0.28
	SE	n.d.	n.d.	n.d.	75.84^{a}±0.91	22.00^{a}±0.00

n.d.: The compound was not detected during the analysis with HPLC.
Different letters in the same column indicate statistically significance between mean values (P<0.05).

So far, yeasts have been successfully used for the purification and removal of mono- and disaccharide by products from carbohydrate preparations (23). Also in this study, the most successive technique for removal of sugars from carob extract was the ethanol fermentation by using *S. cerevisiae*.

Because the yeast preferred using glucose and fructose instead of D-pinitol as carbon source (Table 1). Indeed, fermentation yeasts have been successfully used in the previous studies to remove the sugars from D-pinitol (15, 16, 24).

Besides; although the main aim of this study was not to produce ethanol, after the fermentations, 34.34±2.82 g/L and 57.72±0.42 g/L of ethanol were obtained from the first and second fermentations as by-product. These ethanol concentrations were similar to the studies reported about ethanol production from carob pod (19, 25). However, there was a negative effect of the processes applied before ethanol fermentation step on the ethanol production performance of the yeasts. This might be due to the separation of the possible substrate compounds with the effect of the processes such as clarification and ultrafiltration.

Variation in D-pinitol Contents of The Carob Extracts During The First and Second Processes

The variations in D-pinitol contents of the samples taken after each completed-process were monitored (Table 1, Figure 2). The D-pinitol concentrations of the samples were changed from 8.76 (E1 in Figure 2) to 12.24 g/L EV 1 in Figure 2) and from 12.15 (E2 in Figure 2) to 75.84 g/L (SE in Figure 2) in the first process and second processes, respectively. However, because all the groups had different soluble solids values; all data were normalized according to the lowest soluble solids value in each process (6.08 °Bx for first (E1) and 8.60 °Bx (E2) for second process) to facilitate interpretation of the results (Table 1). Considering the normalized data (Figure 2), the second enrichment process was more successful than the first one in terms of concentration of D-pinitol. Especially ethanol fermentation, ultrafiltration with 5 kDa MWCO and solvent extraction had a significant effect on this increment (Figure 2). The results indicated that both the process type and the process order were very significant for the concentration of D-pinitol from the carob extract.

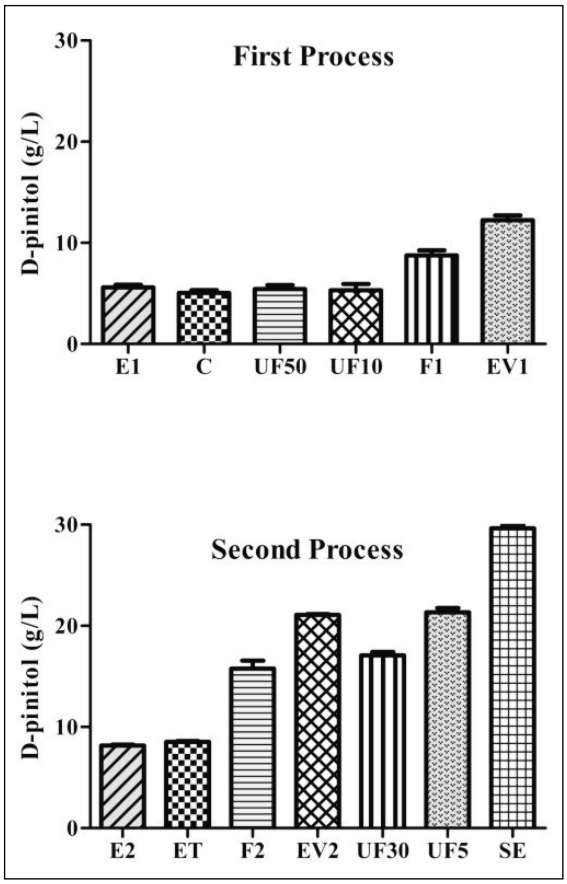

Figure 2. Variation in D-pinitol concentrations from the different stages of the processes (Normalized according the lowest soluble solids value in the same process)

Although the clarification step had no positive impact on the enrichment of D-pinitol, the enzyme treatment (pectolytic and invertase enzymes) was necessary to transform the carbohydrates into an easier form that would be used by the fermentation yeasts as the carbon source. In terms of ultrafiltration; membrane filters higher than 10 kDa MWCO were not effective to separate the D-pinitol compound from the extract. Ultrafiltration can be used for enhancing the D-pinitol concentration of carob extract but its operating order should be after ethanol fermentation. Solvent extraction was the other effective treatment for removal of other impurities from the carob extract (Figure 2).

The influence of the two processes was interpreted by the comparison of D-pinitol concentrations in dry weight basis. Accordingly; the initial and last concentrations of D-pinitol were 8.49 and 21.44 g/100 mL DW for the first and 9.38 and 37.53

g/100 mL DW for the second processes (Figure 3). Results indicated that there was a 2.5-fold increment in D-pinitol concentration in the first process while this increment was 4-fold in the second process.

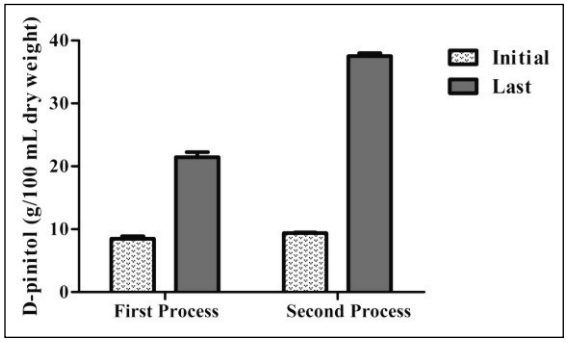

Figure 3. Comparison of the processes in terms of the enrichment of D-pinitol from carob extract

CONCLUSION

The pretreatments are very important if related matrix has a lot of impurities which obstruct the enrichment/purification of a target compound. Many complex separation techniques such as active charcoal column chromatography and ion exchange resins have been defined to obtain D-pinitol products in high purity. However, these techniques are very expensive and difficult to apply. Therefore, pre-purification processes should be in purification steps in order to increase the efficiency of main-purification process/processes.

In this study, different enrichment processes were applied to the carob extract in order to compare their effectiveness on separation of other impurities during the concentration of D-pinitol from carob extract. According to the results; ethanol fermentation, membrane filtration with a membrane filter pore size lower than 5 kDa and solvent extraction were found to be successful for the enrichment purpose when used in combination.

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