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Research Article

Bromelain and Amylase Assisted Extraction of *Cucurbita pepo* Seed Oil Enriched with Phytosterol

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Abstract

Pumpkin (*Cucurbita pepo*) seeds' hydrolysis condition was investigated in order to achieve oil containing high content of phytosterol by using enzyme bromelain and amylase. For this purpose, hydrolysis conditions of bromelain and amylase including ratio of *C. pepo* to water, ratio of concentration of the enzyme bromelain/amylase to substance, temperature, pH, hydrolysis time were investigated.

Bromelain hydrolysis of *C. pepo* seeds with 1.75% enzyme at 55°C for 7hrs at pH of 7 gave 27.83% oil and 1909.95 mg phytosterol in 100 g oil. For amylase hydrolysis these figures were 0.9%; 50°C; 5.5 hrs and 7, respectively; and the oil yield was 14.02% with 2245.03 mg phytosterol in 100 g oil.

Although oil yield by using enzyme bromelain (27.83%) and enzyme amylase (14.02%) was lower than that by using solvent and ultrasonic methods (26.57% to 42.74%), phytosterol content inversely was as 2 to 3 times as higher (631.41 - 772.78 to 19909.95 - 2245.03 mg/100g oil). Fourteen compounds were found in phytosterol mixture obtained by amylase hydrolysis, among them three important e.g. campesterol, stigmasterol, β - sitosterol accounted for 315.460, 120.111 and 189.987 mg/100g oil respectively.

Keywords: Amylase; Bromelain; Oil of Pumpkin Seed; Phytosterol

Introduction

According to FAO statistics, pumpkin area worldwide was more 2.04 million hectares, and the yield reached 13,531 kg/ha in 2018. This data was 444,679 ha in China, 99,510 ha in Turkey and 580,244 ha in India, while those yields were 18,411; 6,192 and 9,599 kg/ha, respectively [1]. In Vietnam, pumpkins are spread across the country because of their high adaptability. Although no specific figures are avaiable, pumpkin production is considered to be potential in agriculture of Vietnam [2].

Phytosterol is one of the most important composition of pumpkin seed with high value biology. Phytosterol content is different from pumpkin seed sources such as 265 mg/100g for *Cucurbita spp.* (United States) in 2005 [3]; 190 - 320 mg/100g oil for *C.pepo* convar *citrullina* (Serbia) in a study of N. Hrabovski., *et al.* in 2012 [4]; 317.2 mg/100g oil for *C. pepo* which reported by Nederal-Nakic'., *et al.* in 2006 [5]. Phytosterol has been used in many enriched functional foods, which is first appeared about twenty years ago and many clinical studies have confirmed its LDL cholesterol- lowing properties [6-8]. There are some products enriched in plant sterols/stanols such as yogurts, milk, spreads and margarines and their beneficial effects have been claimed in clinical studies of Gylling., *et al.* 2014 [9]. Nutritionists have recognized two classes of

phytosterols, there are plant sterols and plant stanols. Plant sterols have a double bond in the sterol ring and the most abundant phytosterols for the human diet are β-sitosterol, campesterol and stigmasterol. The other, plant stanols lack a double bond in the sterol ring, especially sitostanol and campestanol, which comprise only about 10% of total dietary phytosterols [10-12]. Foods or beverages contain at least 1.7g plant sterols, when consumed twice a day for total intake of 3.4g per day help lowering saturated fat and cholesterol and reducing the risk of coronary heart disease (FDA health claim) [13,14].

Soxhlet extraction using traditional solvents such as hexane, petroleum ether or ultrasonic assisted or Bligh and Dyer yielded oil higher than that of conventional leaching [15]. However, the use of solvents has limitations in terms of safety and environmental harms. Therefore, using of biological methods (enzymes), assisting the oil enriched in phytosterol extraction from pumpkin is of interest and application.

Unlike the chemical solvent method, the enzyme method uses water as a special solvent to release compounds out from the mmembrane thanks to the enzyme's hydrolyzation. Due to the hydrophobic properties of oil in water, after enzyme treatment, oil is mechanically separated [16]. Bromelain and Amylase are two common enzymes for cleaving starch and protein in sequence. Using them for hydrolysis helps cleavage the bonds of starch and proteins in the structure of the grain, releasing oil bags. Previously, Kosar Zakeril and partners has found the optimal condition of the hydrolysis process using enzyme Alcalase with E/S = 3%, temperature 44°C for 40 minutes resulted in increasing antioxidant activity of pumpkin seed oil [18].

Aim of the Study

The aim of this study was to use amylase and bromelain for pumpkin seeds treatment to increase phytosterol's content in the obtained oil, which can alternatively replace methods using a solvent.

Material and Methods

Material

Pumpkin seeds (*Cucurbita pepo*) of Vietnam were harvested in the autumn of 2019, dried to a storage humidity of 34.9%.

Bromelain and amylase were purchased from Bio Green –Vietnam and Novo- Denmark, respectively and their specifications are presented in table 1.

Specifications	Bromelain	Amylase
Activity (IU/g)	1200	1300
pH	6 - 8	5 - 9
Temperature (°C)	40 - 60	40 - 80
Substrate	Protein	Starch

Table 1: Specifications of bromelain (Bio Green -Vietnam) and amylase (Novo- Denmark).

Methods

Solvent extraction

Soxhlet extraction: 5g of pumpkin seeds blended for 1 - 2 mm was subjected to Soxhlet extraction using hexane solvent at 55 - 60°C for 7 hours [19].

Bligh and dyer extraction: 100g of pumpkin seeds were blended for 1 - 2 mm, then 200 ml MeOH and 100 ml $CHCl_3$ (v/v = 2/1) were added. Ultrasound assisted this extraction for 2 hours, at 37 KHz. Then, solid phase was subjected to second extraction using100ml $CHCl_3$, ultrasonic assisting for 1 hour. The liquid phases of 2 extractions were combined, the lipid phase was isolated by liquid-liquid extraction, total lipid was obtained after evaporation [20].

Ultrasound assisted solvent extraction:100 grams of ground pumpkin seeds with 300ml hexane solvent was performed in triplicate under ultrasonic waves of 37Khz. Liquid phases were combined and the solvent was evaporated to obtain lipid extraction.

Enzyme assisted extraction

Pumpkin seed/water ratio, enzyme/substrate ratio, temperature, pH and hydrolysis time were investigated at 1/7 to 1/11; 1 to 2% at 0,25% jump; 6 to 8 at 0,5 jump; 40 to 60°C at 5°C jump; 4 to 8 hours, at 1 hour jump, respectively for bromelain; while those were: 1/5 to 1/9; 0.7 to 1.1% at 0.1% jump; 5 to 8 at 1 jump; 40 to 80°C at 10 °C jump; 4 to 6 hours at 0.5 hour jump, respectively for amylase.

The activity of enzyme bromelain and amylase is expressed by dissolved protein content and by reducing sugar content, respectively.

At the end of enzymatic hydrolysis, oil-enriched phytosterol was obtained by centrifugation at 7000 rpm for 5 minutes.

Total protein content analysis

Total protein content was determined by Bradford assay, which relies on the binding of the dye Coomassie Blue G250 to protein. The result is achieved by measuring the absorbance of the solution at 595 nm and comparing with standard curve using the formula: [21]

$$y = \frac{x-0.2238}{0.2237}$$

Where: x: Optical adsorption (OD) at 595 nm.

y: Protein concentration of sample (mg/ml).

Total reducing sugar content analysis

DNS (Dinitrosalicylic acid) is one of the reagents used to estimate reducing sugars in the solution. 3,5- Dinitrosalicylic acid is reduced to 3 amino 5 nitro salicylic acid while oxidizing the reducing sugars. The color change can be quantified spectrophotometrically at a wavelength of 540 nm and the total sugar content is determined by the followings [22]:

$$y = \frac{x-0.3883}{0.0443}$$

Where: x: Optical adsorption (OD) at 540 nm.

y: Reducing sugar concentration of sample (µg/ml).

Total lipid content analysis: The lipid content was analyzed by the Soxhlet method [19].

Total phytosterols content analysis

Blended pumpkin seeds after 1extraction, 1 ml lipid phase was collected by centrifugation at 7000 *rpm* then evaporated. Phytosterol content was determined equivalent to β-sitosterol by ultraviolet-visible spectrophotometer.

$$y = Cs * Aa/As * n * 100$$

Where:

C_s: Standard concentration

A_a: Absorbance of the sample; A_s = Absorbance of the standard

y: Reducing sugar concentration of sample (µg/ml)

n: Dilute coefficient of sample

100: Coefficient for 100 ml oil pumpkin seed extraction..

Phytosterol composition

Pumpkin seed oil obtained by amylase assisted extraction was treated with the ratio of KOH 5% to oil of 2,6; at 74°C for 4hrs for phytosterol enrichment. Then the sample was transformed into a derivative of trimethylsilyl (TMS) at 70°C for 2 hours. The extract with n-hexane was evaporated then analysed on gas chromatograph Thermo Finnigan Italia S.P.A. TRACE GC Ultra series, equipped with DB column 30m x 0.53 mm, film 0.5 µm, the program ran from 200°C, 2°C/ min, to 300°C then kept for 10 minutes, carrier gas, flow rate 10ml/minute. The phytosterols will be compared with previously run standards to identify each ingredient and quantify with the calibration curve of each substance.

Results and Discussion

Cucurbita pepo's seed composition

Cucurbita pepo seed's composition is presented in table 2. Oil content was of 42.74% which was of the same range with *C. pepo* from Serbia and higher than that of *C. maxima* of Bangladesh. Phytosterol content was 2705.76 mg/100g oil, much higher than that was found in Serbia sample. Protein (36.42%) and starch (10.08%) accounted for 81.15% of non-oil content of pumpkin seed, therefore, using bromelain and amylase for hydrolysis helps cleavage the bonds of starch and proteins in the structure of the pumpkin to release oil and phytosterol more effectively.

	***Cucurbita pepo*, Vietnam**	***Cucurbita pepo*, Serbia [4]**	***Cucurbita maxima*, Bangladesh [23]**
Moisture (%)	8.02	9.20	4.06
Ash (%)	4.28	-	3.80
Total protein (%)	36.42	-	2.15
Sugar (%)	10.08	-	
Starch (%)	9.07	-	34.56
Total oil (%)	42.74	43.37	36.70

Table 2: Pumpkin seed composition.

Cucurbita pepo's seed treatment with bromelain and amylase was investigated in term of seed to water ratio, enzyme to seed ratio, pH, temperature, and time. The results were expressed in protein (mg/µg) for bromelain or reducing sugar (mg/µg) for amylase treatment (Figure 1).

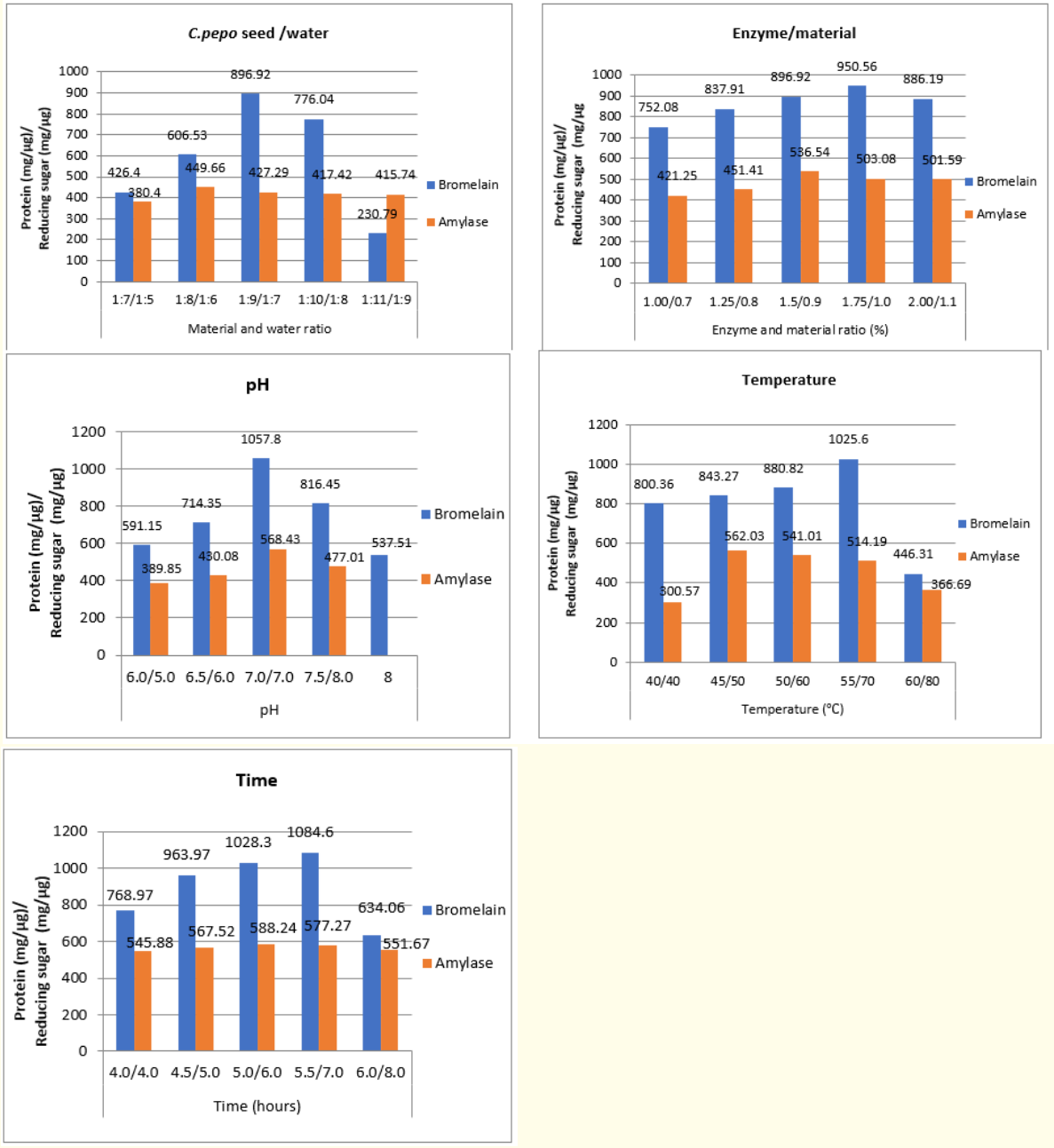

Figure 1: *Cucurbita pepo* seeds treatment by bromelain and amylase.

In the enzymatic hydrolysis, water was added at an appropriate ratio for enzyme activation, while temperature influenced on the diffusion rate and viscosity reduction. Results are figured out in figure 1, in which the suitable condition for *C. pepo* seeds treatment by bromelain were: *C. pepo* seed/water = 1/9, bromelain/material = 1.75%, at 55°C, pH = 7 for 7 hours. At this treatment the protein released highest at 1025.66 mg at 55°C before reducing to 446.31 mg when hydrolysis temperature was 60°C. For amylase, those were: *C. pepo* seed/water = 1/6, amylase/material = 0.9%, at 50°C, pH = 7 for 5.5 hours and reducing sugar content released as highest as 588.24 µg.

Hexane extraction, bligh and dyer extraction, ultrasound assisted hexane extraction, enzyme assisted extraction

The performance of hexane extraction (55 - 60°C, in 6 hours); Bligh and Dyer extraction (CH_2Cl_2/MeOH (2/1) at 45 - 50°C, in 3 hours); ultrasound assisted hexane extraction (ultrasonic waves of 37Khz, at 45 - 50°C, in 3 hours); enzyme assisted extraction (With bromelain: *C. pepo* seed/water = 1/9, enzyme/material = 1.75%, at 55°C, pH = 7, in 7 hours and with amylase: *C. pepo* seed/water = 1/6, enzyme/material = 0.9%, at 50°C, pH = 7, in 5.5 hours) is presented in table 3 in term of oil recovery (%), oil yield (g/100g material), phytosterol content (mg/100 ml oil).

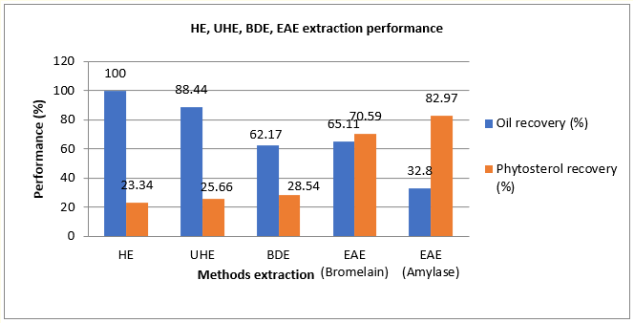

Figure 2: Oil and phytosterol extraction performance.

Extraction performance	HE	UHE	BDE	EAE	
				Bromelain	Amylase
Oil recovery (%)	100	88.44	62.17	65.11	32.80
Oil yield (g/100g material)	42.74	31.62	26.57	27.83	14.02
Phytosterol content (mg/100 ml oil)	631.41 ± 2.88	694.24 ± 2.53	772.78 ± 3.65	1909.95 ± 3.34	2245.03 ± 3.52

Table 3: HE, UHE, BDE, EAE extraction performance.

HE: Hexane Extraction; UHE: Ultrasound Assisted Hexane Extraction; BDE: Bligh and Dyer Extraction; EAE: Enzyme Assisted Extraction.

Oil recovery is highest in hexane extraction. Phytosterol yield (mg/100g material) were 269.84; 219.52; 205.31; 531.54 and 314.75 for HE, UHE, BDE, bromelain EAE and amylase EAE, respectively. Phytosterol (mg/100g oil) in pumpkin seed (*C. pepo* convar *citrullina*) oil extracted by hexane, petroleum ether and supercritical CO_2 claimed by Natasa Hrabovski and partners 2012 [20] were in the same range of 225, 244 and 294, respectively. The difference in phytosterol extraction could be explained with difference in extraction kinetics of phytosterol and triglycerides in plant cell structure. Using enzymes, the sterols and stanols are extracted more easily and effectively. Thus, the phytosterol content (mg/100 ml oil) were 631.41; 694.24; 772.78; 1909.95 and 2245.03 for HE, UHE, BDE, bromelain_EAE and amylase_EAE, respectively. Among bromelain and amylase, amylase assisted extraction gave less oil yield but better in phytosterol enrichment.

Phytosterol composition

The composition of Phytosterol is presented in the table 4.

Phytosterol	Content (mg/100g pumpkin seed oil)	Reference (mg/100g pumpkin seed oil) [17]
Cholesterol	20.796	9.21
Brassicasterol	42.466	11.32
24-Methylenecholesterol	34.635	9.30
Campesterol	315.46	82.46
Campestanol	36.335	8.08
Stigmasterol	120.111	97.71
Δ^7-Camersterol	36.114	32.44
$\Delta^{5,23}$ Stigmastadienol	24.446	21.57
Chlerosterol	720.325	525.91
β - Sitosterol	189.987	264.25
Sitostanol	111.422	112.67
Δ^5-Avenasterol	76.356	15.92
$\Delta^{5,24}$-Stigmastadienol	455.678	394.18
Δ^7-Avenastenol	76.222	34.09
Total Phytosterol	2260.353	1619.55

Table 4: The composition of phytosterol.

Three important compositions of phytosterol e.g. campesterol, stigmasterol, β - sitosterol accounted for 315.460, 120.111 and 189.987 mg/100g oil respectively.

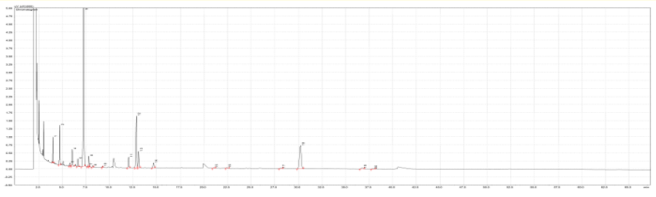

Figure 3: Chromatogram of phytosterol on DB-5 column.

Conclusion

Bromelain assisted extraction at 55°C, pH = 7, for 7 hours with *C. pepo* seed/water = 1/9, Enzyme/material = 1.75% yielded phytosterol concentration in oil of 1909.95 mg/100 ml oil, while amylase assisted extraction at 50°C, pH = 7, for 5.5 hours with *C. pepo* seed/water = 1/6, Enzyme/material = 0.9%, yielded phytosterol concentration in oil of 2245.03 mg/100 ml oil. Hexane, Bligh and Dyer extraction and ultrasonic assisted hexane extraction were carried out for comparison, and their phytosterol content in 100 ml oil were about 3 times lower than that performed by enzyme assisted extraction. Bromelain and amylase assisted extraction improved the phytosterol concentration in the oil obtained, where amylase gave less oil yield but better in phytosterol enrichment. The oil yield of this method is lower than that of solvent and ultrasonic extraction, therefore, *C. pepo* seed after enzyme extraction is recommended for second solvent extraction to obtain more oil. The extracted phytosterol consisted of 14 components in which campesterol, stigmasterol, β - sitosterol accounted for 315.460, 120.111 and 189.987 mg/100g oil respectively.

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