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Aqueous Enzymatic Extraction of Oil and Protein Hydrolysates from Peanut

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Aqueous enzymatic extraction (AEE) is a safe and efficient vegetable-oil extraction process that may also result in edible protein hydrolysates. In this study, an AEE process was developed to recover oil and protein hydrolysates from blanched peanut. The enzyme type and enzyme concentration for the most efficient extraction were selected. Under the condition of pH8.50, 60oC, an enzyme level of 1.5% and 8 h incubation, peanut protein hydrolysates (PPH) yield of 82.5% and oil yield of 92.2% were achieved when using alcalase2.4L. The fatty acid composition of oil obtained by AEE vs. hexane-extraction was very similar. The good oil quality could save the refine cost for edible. The functional properties of PPH were first investigated and they showed good effects of scavenging DPPH (α- dipheny -β- picrylhydrazyl) free radical and inhibiting the angiotensin-I-convertine enzyme (ACE).

Keywords: aqueous enzymatic extraction, peanut, free oil, protein hydrolysates

Introduction

Peanut seeds contain 24-28% (w/w) protein and 45-52% (w/w) oil. Conventional process to extract oil from peanut includes mechanical pressing and solvent extraction. Mechanical pressing is a less efficient process, leading to low oil recovery (40-60%) and bad protein denaturation (Aparan *et al*., 2002). Solvent extraction, although giving high oil recovery (90-98%), has inherent disadvantages of poor quality of protein in the residual meal, high investment and energy requirements (Barrios *et al*., 1990). The commercial hexane used as the most common solvent for oil extraction is listed among hazardous air pollutants associated with neurological and respiratory disorders on prolonged exposure (Marlowe *et al*., 1991).

Aqueous enzymatic extraction (AEE) is an eco-friendly; alternative process based on simultaneous isolation of oil and protein from most oilseeds. The differences in the oilseed composition determine the choice of enzymes to be used for each oilseed. The role of most carbohydrate enzymes such as cellulases and pectinases in AEE is to break the structure of cotyledon cell walls and the action of the proteolytic enzymes is to hydrolyze the protein in the cell membrances as well as the inside cytoplasm. In the case of peanut, the average sizes of protein bodies and lipid bodies in cotyledon cell are 8-10μm and 1-2μm. The spaces between protein bodies are filled with the lipid body and cytoplasmic network which presumably composed of protein (Young and Schadel, 1990). Lanzani *et al.* (1975) attempted to extract peanut oil by AEE and they got 72% oil recovery by aqueous extraction. But use of four kinds of enzymes increased oil yield, at the most, by 6%. Aparna and others (2002) used papain, trypsin, chymotrypsin and Protizyme™ separately and obtained the oil yield of 76%, 61%, 67% and more than 86% respectively. These works stressed selecting a more effective enzyme to oil recovery and adopted high speed centrifugation (18,000 × g). Moreover, till now there were no reports about the recovery of the resulting and the functional properties of PPH.

In recent years, the search for functional components in food has become a major area of research. Bioactive peptides are a group of functional compounds and they have beneficial effects for the organism. Oilseed as are attracting increasing as a source of edible proteins. These proteins could find applications in the food industry if appropriate hydrolysis processes are applied (Radha *et al*., 2007). Peptides with antioxidation and antihypertensive activity have been obtained from soybean (Wu and Ding, 2001) and wheat (Matsui *et al*., 1999). But there is no research about peptides

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generated by peanut. The objective of this study is to investigate the effect of different enzymes on the extraction yield of free oil and PPH based on the suiting AEE process, compare the quality of oil by AEE with the oil by solvent extraction. At the same time, the PPH were recovered and concentrated and the antioxidant activity and ACE inhibition of the PPH were evaluated.

Materials and Methods

Materials Blanched peanut beans were supplied by Wu.L Food Company (Shandong Province, China) and stored at 4°C until used. The composition (g/100g dry matter) was 49.32% oil, 25.43% protein, 5.12% fiber and 3.54% ash. Alcalase2.4L (EC 3.4.21.62, *Bacillus Licheniformis*) was purchased from Novozymes (Novo, China). Neutral As1398 (EC 3.4.21.14, *Bacillus subtilis*) and papain (EC 3.4.22.2, Papaya Latex), were bought from Genencor (Wuxi, China). Protease-N (EC 3.4.24.28, *Bacillus subtilis*) was provided by Amano Enzyme China LTD. Hippuryl-histidryl-L-leucine (HHL) and ACE were from Sigma Chemical Company. DPPH was of analytical grade from Nacalai Tesque (Japan).

AEE process Experiments were carried out in a 2L reactor connected with a thermostatically controlled water bath. For each experiment, 300g blanched peanut was ground by a muller (Type6202, made in China) at high speed. The ground peanuts were transferred to the reactor and dispersed in distilled water at 1:5(wt/vol) seeds-to-water ratio by gentle stirring to make slurry. The slurry pH was adjusted to 8.50 by adding 1 mol/L NaOH and incubated at 60°C for 30 min with continuous stir at 200rpm in order to solubilize more protein in the aqueous phase and hence enhance the protease hydrolysis and protein extractability. The protease was added after the pH and temperature of the slurry were adjusted to the optimal condition of different enzymes. The mixture was then incubated for 8h with continuous stir followed by centrifugation at 1,819 × g (3000rpm) for 20 min to obtain free oil, aqueous phase, emulsion and solid phase. The upper oil phase named free oil (I) was collected with a pipette. The solid phases was dispersed into 600mL water again and incubated at 60°C for 30 min and then centrifuged as above to obtain the aqueous phase and the residual meal. The aqueous phase from twice centrifugation were combined, heated to 85°C and kept for 10 min in order to inactive the protease, and then centrifuged again to obtain the PPH solution. The PPH solution was concentrated by nanofiltration (NF) with crimped membrane featuring NF200-2540 aroma polyamide with a surface area of 2.6 m^2 and a nominal molecular-weight cut-off of 150Da and spray-dried with inlet temperature 175-185°C and outlet temperature 75-85°C and the PPH powder was obtained for further study. The emulsion from the first centrifugation was transferred into centrifuge tubes, frozen at -16°C for 16 h and thawed at 35°C for 1 h, then centrifuged at 3234 × g (4000rpm) for 20 min. The free oil (Π) from top of centrifuge tubes after de-emulsification was collected. A control without enzyme was also carried out. All extraction experiments were performed at least two times and mean values of the data are reported.

Extraction yields of PPH and free oil The PPH extraction yield and free oil extraction yield were expressed as follows:

PPH yield, % = protein in PPH solution × 100/total protein in blanched peanut

Free oil yield, % = [free oil (I) + free oil (Π)] × 100 / total oil in blanched peanut

n-Hexane-extracted oil The blanched peanut were manually flaked to 0.1-0.2mm thickness. Crude oil in flaked peanut was extracted with commercial n-hexane by Soxhlet extractor (Soxtex Avanti 2050, Foss Co.Damark) for 90 min. The miscella was collected and evaporated in a rotary evaporator at 40°C for oil quality analysis.

Analytical methods Protein content was calculated as Kjeldahl Nitrogen AOAC 960.52 (%N×5.46). The oil content of the blanched peanut was determined by AOAC960.39. The physicochemical properties of all oils in terms of specific gravity, iodine value, free fatty acid (FFA) content and peroxide value were analyzed by AOAC methods (AOAC, 1990). All experiments were carried out in triplicate.

Degree of hydrolysis (DH) measurement of peanut protein The DH values, defined as the percentage of the total number of peptides bonds in a protein which have been cleaved during hydrolysis (Adler-Nissen,1986), were quantified with the method described by Spellman *et al.* (2003) using picrylsulphonic acid (TNBS). The higher DH value represents the deeper extent of protein degradation.

Determination of Fatty acid (FA) composition The FA composition of oil was determined with a gas chromatograph (model GC-14B, Shimadzu Co. Japan) equipped with a flame-ionization detector and a SPB-5 column (30 m, 0.25 mm i.d., 0.25µm film thickness; Supelco, USA). Oil dissolved with n-hexane was flowed with a column packed with neutral alumina to separate the free fatty acid. After evaporation under s steam of nitrogen, the collected oil were hydrolyzed and methylated by the sodium hydroxide and boron trifluoride. Prepared esters were extracted with n-heptane and 0.5 µL of the upper phase was injected into the GC. The carrier gas was nitrogen at a flow rate of 30 mL/min. Injection temperature was 230°C, Column temperature was programmed at 5 °C/min from 100°C to 230°C. The fatty acids were identified with reference to the retention times of esters of standard fatty acids that were under similar conditions.

Analysis the DPPH free radical scavenging effect The scavenging effect of the hydrolysates on DPPH free radical was measured by Wu and others (2003) with some modifications. A volume of 0.2mL sample (with different concentration PPH) was added to 5.5mL 65 ug/L DPPH in ethanol. The mixture was shaken and then left undisturbed for 30min at room temperature, and the absorbance of the resulting solution was measured at 520nm and noted as A_s. The absorbance of the mixture of 5.5mL ethanol and 0.2mL sample was measured and noted as A_j. The absorbance of the mixture of 5.5mL DPPH in ethanol and 0.2mL water was measured and noted as A_o. A lower absorbance represented a higher DPPH scavenging activity.

$$\text{The scavenging effect (\%)} = [1 - \frac{A_s - A_j}{A_o}] \times 100\%$$

Measurement of ACE inhibitory activity The ACE inhibitory activity assay was performed using a modified version of the method of Wu *et al.* (2002). A sample (10μL, with different concentration PPH) and 40μL HHL (2.17mmol/L HHL in 50mmol/L sodium borate buffer, pH8.3, containing 300mmol/L NaCl) as a substrate were preheated at 37°C for 6 min. The reaction was started by addition of 20 μL ACE (0.1unit dissolved in the same buffer). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 1mol/L HCl (80μL). 10μL of the reaction mixture were injected in a Water 2690 separation Module (Waters, USA) on a Symmetry C18 column (3.9mm×150mm, Pharmacia) using 12% acetonitrile in the presences of 0.5% acetic acid as the mobile phase at 30°C at a flow rate of 0.5mL/min. The inhibition activity is related to the elution peak geometry of Hipputic acid liberated by the action of ACE using the relationship (1), Where P_B is the peak area without PPH, P_S is the peak area of the reaction mixture with PPH solution.

$$\text{ACE inhibition activity (\%)} = \frac{P_B - P_S}{P_B} \times 100\% \qquad (1)$$

Results and Discussion

Effect of enzyme type on free oil and PPH yields The effect of using enzymes was quite drastic and it was clear that the free oil and PPH yields obtained with enzymatic treatment were much higher than that of the control and each type of enzyme worked efficiently (Tab.1). Without using enzyme, free oil yield was only 72.5%, while alcalase increased the free oil yield to about 92%. Lanzali obtained oil yields of 75% using 3% cellulase; the extraction without enzymes resulted in a 72% yield. The protease were more effective than the carbohydrate enzymes reported by Lanzali *et al.* (1975), most likely because of the high protein content of the peanut (25.19%), resulting in a greater association between fats and protein in the cell and in the cells membrance than the association of fats with other components of the seed. Proteolytic enzymes can break down the lipid body membranes due to hydrolysis of the oleosins and disrupt the cytoplasmic network which is largely composed of protein (Rosenthal *et al.*, 1996), thereby making the inner structure less tightly bound and compact and thus enabling easier removal of protein and oil from the cell.

The type of enzyme used was found to increase not only the oil yield, but also PPH yields distinctly under their own optimal enzymatic conditions with the same enzyme con-

Table 1. Effect of different enzymes on the free oil and PPH yields.

Commercial name	pH* used	Temperature* Used(°C)	Enzyme activity (U/mg enzyme)#	DH (%)	Free oil yield (relative %)	PPH yield (relative %)
Control	8.0	60	----	0	72.5±1.4	0
As1.398	7.5	55	320	13.2	85.9±0.97	75.2±0.94
Alcalase	8.5	60	300	22.6	91.7±1.3	85.9±1.1
Papain	7.0	50	178	12.3	84.5±2.0	72.4±0.67
Protease-N	7.5	55	254	17.4	86.9±1.9	78.1±1.0

*pH and temperature were adjusted to each enzyme's optimum as specified by the suppliers.

The enzyme activities were determined under their optimum conditions respectively. One unit (U) is the amount of enzyme digests casein at an initial rate that produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as 1 μg tyrosine released per min.

centration (v or g /100g protein weight, 1.5%) and incubation time (8 h) .The oil yield and PPH yield were increasing with the increase of the DH. Although the specific activity of As1.398 was the highest, the yields were last but one. The reason might be the peanut protein that is not an appropriate substrate for As1.398. Yoon *et al.* (1991) also found that different proteases at the same enzyme/substrate ratio had different effect. The yields were highest when alcalase was used as compared As1.398, papain and protease-N. It was similar with the result reported by Hanmoungjai *et al.* (2001) that alcalase was useful for aqueous enzymatic extraction of oil from rice bran. Alcalase is an endoproteinase with broad specificity and has been reported to cleave the peptide bonds at Gln^4-His^5, Ser^9-His^{10}, Leu^{15}-Tyr^{16} and Tyr^{26}-Thr^{27} (Ottesen and Svendsen, 1970). The stronger effect of alcalase compared to other enzymes could be attributed to the hydrolysis of the membranes surrounding lipid bodies and the cytoplasmic protein more effectively and the production of more small peptides having a great higher solubility. These effects allow not only the extraction of the nitrogenous compounds of proteinaceous origin, but also an increase of the extraction yield of oil. This hypothesis is supported by the DH values of the system hydrolyzed by different proteases. It reached 22.6% when alcalase was used and it is almost twofold of that of papain.

Determination of the enzyme concentration of Alcalase 2.4L The alcalase concentration required for optimal recovery of free oil and PPH was also determined by using enzyme levels of 1.0, 1.5, 2.0, 2.5% and 3% (volume of enzyme/weight of the peanut protein). All the experiments were run in duplicate, and the difference in the individual values was less than 3%. Fig.1 shows the relationship of the free oil yield and PPH yield with the enzyme levels under the optimum temperature and pH at 8h. The PPH yields were increased when the enzyme concentration was increased from 0.5% to 3% (v/w). The reason could be explained by the better solubility and more deeply hydrolysis of peanut protein as the enzyme levels increasing. It was confirmed by the curve of DH with enzyme concentration. There was a gradual increase in DH with the increase in the enzyme concentration from 0.5% to 3% and more soluble peptides were released. The highest oil yield was obtained with 1.5% enzyme. Very litter decrease in oil yield occurred when enzyme levels were further increased to 3%. The main reason is when higher enzymes level was adopted, the higher DH and more free amino acids were obtained, and it caused the pH of incubation system became lower and part of oil were sediment with the large molecular protein that was produced by one-by-one mechanism (Jens, 1986). The results demonstrated that a dose higher than 1.5% (v/w) of enzymes is unnecessary for oil recovery. At 1.5% for 8 h incubation, the recovery yield of PPH was 82.2% and the free oil yield was 92.2%. The yields of oil and PPH are higher than the results reported by others.

Physicochemical properties of extracted oil The oil extracted by AEE was clear and bright yellow in color. Table 2 shows the qualities of oils by AEE and by solvent process. The data of the oil characteristic were very close in term of specific gravity, refractive index and iodine value. However, the FFA content and phosphatides content of oil obtained by

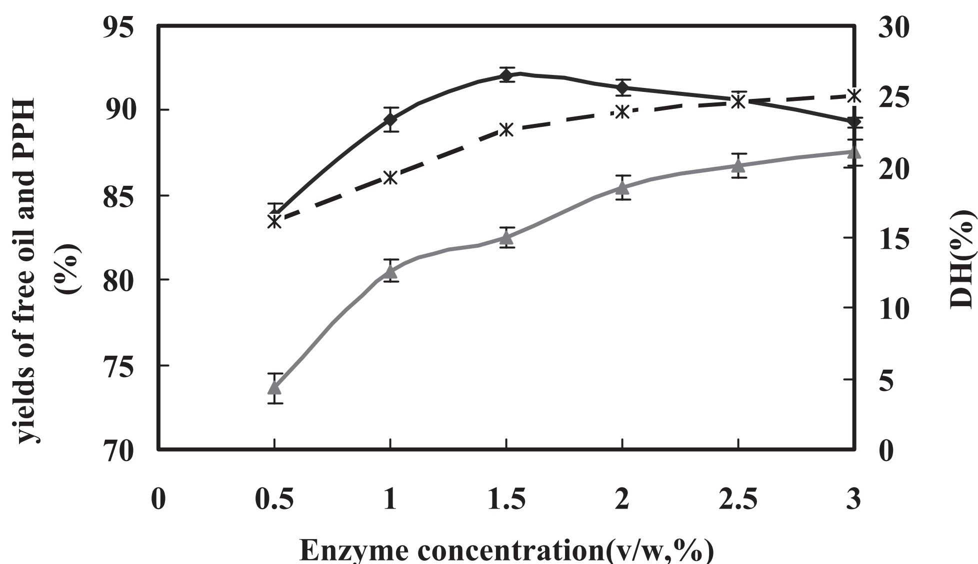

Fig. 1. The relationship of the yield of PPH and free oil and DH with the enzyme levels. The symbols ▲, ● and ⁎ represent the PPH yield, free oil yield and DH.

Table 2. Quality of peanut oil obtained by different processes.

Analytic characteristic	Commercial edible oil by solvent extraction[a]	AEE oil[b]	n-Hexane extraction oil[b]
Specific gravity (20°C)	0.914-0.917	0.916	0.916
Refractive index(20°C)	1.460-1.465	1.470	1.469
Iodine value	86-107	93.7	93.4
Moisture and volatile (%)	≤ 0.10	0.094	0.102
FFA (g oleic acid/100g)	≤ 0.5	0.197	0.979
Peroxide value(mmol/L)	≤ 7.5	1.14	1.03
Phosphatides content	----	0.22	0.34

[a] Date from China peanut oil standards (the third class), GB1534-2003.

[b] Values present the means of three determinations.

Table 3. Fatty acids composition of the peanut oil extracted by different extraction methods.

Fatty acid composition (%)	Codex Alimentarius Standard[a]	Methods of extraction	
		Aqueous enzymatic extraction[b]	n-Hexane extraction[b]
palmitic(C16:0)	8.0-14.0	11.3±1.76	10.7±0.92
stearic (C18:0)	1.0-4.5	3.5±0.23	3.8±0.71
oleic (C18:1)	35.0-67.0	46.7±2.51	47.7±2.79
linoleic (C18:2n-6)	13.0-43.0	31.9±3.12	31.2±2.61
arachidic (C20:0)	1.0-2.0	1.8±0.98	1.7±0.15
behenic(C22:0)	1.5-4.5	2.5±0.11	2.3±0.33
Eicosenoic (C20:1)	0.7-1.7	1.6±0.01	1.6±0.10
lignoceranic(C24:0)	0.5-2.5	1.1±0.02	1.3±0.08

[a] date from Codex Alimentarius Standard 210-1999.

[b] All the analyses were done in triplicate and are reported as mean±SD

AEE were significantly lower than n-hexane extracted oil, which suggested a lower cost in the refining stage for edible and the possibility of employing physical refining (Bocevska *et al.*, 1993). Although the peroxide value of AEE oil is higher than n-hexane extraction oil, it was below the threshold value of the commercial edible oil. Results obtained in this work agree with previous reports, where the AEE oil had lower FFA levels and phospholipids content (Robert *et al.*, 2004; Bocevska *et al.*, 1993).

The FA compositions of peanut oil extracted by AEE and hexane-extracted are presented in Table 3. The FA profile of oil is not modified when using different processes and they are comparable. This result is similar to one in which enzymatic extraction were used (Concha *et al.*, 2006). The

predominant fatty acids are stearic, oleic, linoleic, palmitic, arachidonic, arachidic, behenic and lignocerane acids and oleic acid was the most prominent FA.

The antioxidant activity and the ACE inhibition of PPH DPPH is a stable free radical that has a maximum absorbance at 520nm in ethanol. When DPPH encounters a proton-donating substance, the radical would be scavenged and the absorbance would be reduced (Shimada *et al.*, 1992). Based on this principle, the antioxidant activity of the substance can be scaled with its ability in scavenging the DPPH radical. Fig.2 shows the hydrolysates have the ability to quench the DPPH radical. The scavenging effect of the hydrolysates was increased gradually with the hydrolysates concentration. More than 80% of DPPH radical was scavenged at 50mg/ml, which was a litter higher than that of protein hydrolysate from yellow stripe trevally protein (Klompong *et al.*, 2007) and a litter lower than that of protein hydrolysates prepared from round scad (Thiansilakul *et al.*, 2007). The results revealed that the hydrolysates were electron donors and could react with free radicals to terminate.

Recently the study of ACE has attracted a great deal of interest. A very important group of peptides is the ACE inhibitors that are currently in use as antihypertensive agent. It is apparent that there was a concomitant increase in ACE inhibition with increasing concentrations of the sample (Fig.3). At first the ACE inhibition increased fast with the increasing concentrations at the range of 0.2mg/mL to 1mg/mL. The ACE inhibition activity reached 67% at 1mg/ml. When the

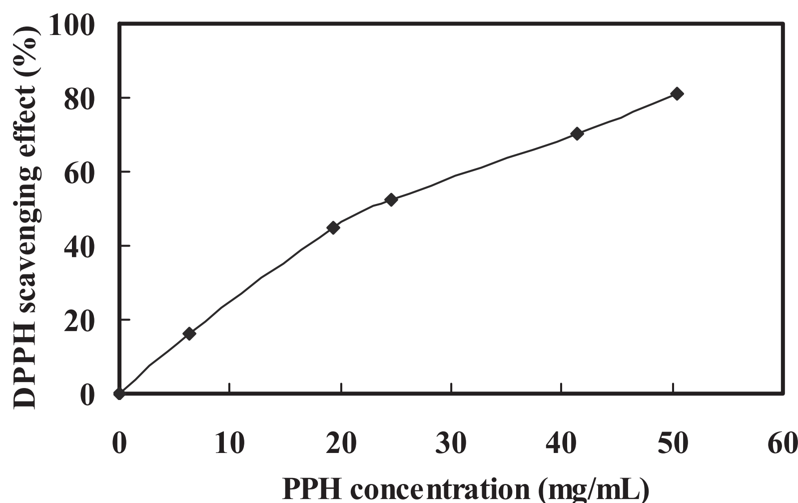

Fig. 2. The scavenging effect on DPPH radical by PPH at different concentration Each value is expresses as the mean (n=3).

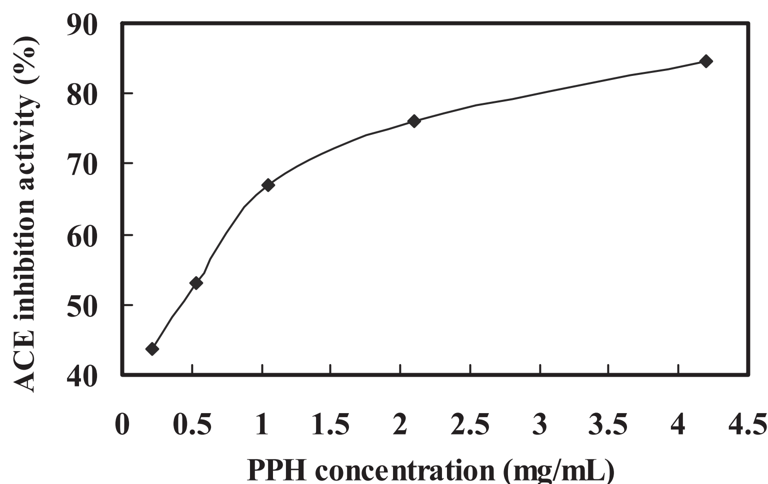

Fig. 3. The ACE inhibition activity of PPH at various concentrations.

concentration was more than 1mg/ml, the ACE inhibition increased slowly. Chiang *et al.* (2006) got the similar result that the ACE inhibition increased fast with the increasing concentrations at initial concentration and then slowly with the higher increasing concentrations when they using different enzymes to produce soy protein hydrolysate with ACE inhibition. And the ACE inhibition activity was 50% at 1.79mg/ml soy protein hydrolysate. Meisel and others (1999) suggest that the hydrophobic residues tryptophan, proline and tyrosine as well as arginine are responsible for ACE inhibition when present at the C-terminal of the peptides. The fact that the enzymatic hydrolysates possessed ACE inhibition activity may be associated with the exposing of more hydrophobic residues.

Conclusions

Using alcalase could increase the free oil yield greatly compared without using enzyme. The quality of the oil obtained by AEE is the same as or better than that of n-hexane extracted oil. The resulting hydrolysates recovery could improve the merit of the process. The hydrolysates, which offset some disadvantages of the peanut protein such as lack of some limited amino acids, have the effects of scavenging DPPH radical and inhibiting ACE. All of these functions will expand the application field of the hydrolysates. In a word AEE is likely to be a viable technology for oil extraction in the future.

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