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Optimization of the Aqueous Enzymatic Extraction of Rapeseed Oil and Protein Hydrolysates

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Abstract An aqueous enzymatic extraction method was developed to obtain free oil and protein hydrolysates from dehulled rapeseeds. The rapeseed slurry was treated by the chosen combination of pectinase, cellulase, and β-glucanase (4:1:1, v/v/v) at concentration of 2.5% (v/w) for 4 h. This was followed by sequential treatments consisting of alkaline extraction and an alkaline protease (Alcalase 2.4L) hydrolysis to both produce a protein hydrolysate product and demulsify the oil. Response surface methodology (RSM) was used to study and optimize the effects of the pH of the alkaline extraction (9.0, 10.0 and 11.0), the concentration of the Alcalase 2.4L (0.5, 1.0 and 1.5%, v/w), and the duration of the hydrolysis (60, 120, and 180 min). Increasing the concentration of Alcalase 2.4L and the duration of the hydrolysis time significantly increased the yields of free oil and protein hydrolysates and the degree of protein hydrolysis (DH), while the alkaline extraction pH had a significant effect only on the yield of the protein hydrolysates. Following an alkaline extraction at pH 10 for 30 min, we defined a practical optimum protocol consisting of a concentration of 1.25–1.5% Alcalase 2.4L and a hydrolysis time between 150 and 180 min. Under these conditions, the yields of free oil and protein hydrolysates were 73–76% and 80–83%, respectively. The hydrolysates consisted of approximately 96% of peptides with a MW less than 1500, of which about 81% had a MW less than 600 Da.

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Introduction

Rapeseed (Cruciferae family) is one of the most important oilseeds in the world, ranking fourth with respect to production after soybean, palm, and cottonseed [1]. In China, the production of rapeseed exceeds 12,000,000 tons per year. Rapeseed contains high-quality oil (38–46%) and protein (20–30%) as well as some antinutritional compounds, such as glucosinolates, phenols, and phytic acid [2]. Conventional industrial processing of rapeseed involves pressing and hexane extraction, which yields two products – the oil and a low-valued meal that is mainly used as animal feed or fertilizer.

The use of hexane for extracting rapeseed oil has recently become the focus of concerns with respect to its safety and environmental effects; these concerns have increased following the listing of hexane among the group of hazardous air pollutants [3]. An alternative process, aqueous (enzymatic) extraction, for extracting oil from many oil-bearing seeds/fruits has been attempted in the laboratory and/or at the pilot industrial scale level [4, 5]. The process usually consists of an aqueous (enzymatic) extraction of the comminuted materials, followed by a centrifugal separation of the slurry into oil, emulsion, and the aqueous and solid phases. Protein may be recovered in the aqueous or solid phase, depending on the conditions selected [6]. Compared with the traditional technology, this process is mild and safe due to the complete avoidance of

organic solvents. Despite these advantages, this novel method has met with some problems, such as fairly low oil yield and the formation of an emulsion that necessitates a mechanical demulsification operation in the downstream process [5]. Demulsification is generally not easy; as such, it may become a bottleneck in the development of an aqueous enzyme extraction technology. Indeed, large protein molecules contribute much to the undesirable stability of the resulting emulsions. Olsen [7] used a protease (Alcalase 0.6L) to simultaneously produce soy protein hydrolysates and recover the oil from fat-containing soy material. Recently, Alcalase has also been used to hydrolyze rapeseed protein isolates, and the protein hydrolysates were observed to have special bioactivity such as a source of human immunodeficiency virus (HIV) protease peptide inhibitors [8]. Alcalase can, therefore, be considered for use following carbohydrase treatment of rapeseeds to hydrolyze the protein, break the emulsion, and simultaneously obtain free oil as well as protein hydrolysates in the aqueous extraction system.

In rapeseed, some attempts aimed at aqueous extraction of oil with enzymes have also been reported. Lanzani et al. [9] obtained a rapeseed oil extraction yield of 78% with the combination of protease and α-1, 4–galacturonide glycano-hydrolase enzymes. Fullbrook [10] reported that the highest oil recovery was 72% when crude enzyme preparations from *Aspergillus* sp. were used during the aqueous hydrolysis of rapeseed slurry in the presence of hexane. Jensen et al. [11] used a multi-activity enzyme, SP-311, to extract rapeseed oil and obtained oil, a dehulled protein-rich meal with a low content of antinutritional substances, and syrup after three washings and centrifugation steps. However, none of these studies reported further utilization of the rapeseed protein.

In the investigation reported here, Alcalase 2.4L was used following the aqueous carbohydrase treatment of dehulled rapeseeds for the simultaneous recovery of the free oil and protein hydrolysates. The effects of different commercial carbohydrases on the extraction of emulsified oil were studied initially. This was followed by an optimization of the Alcalase 2.4L hydrolysis process by means of response surface methodology (RSM).

Materials and Methods

Materials

Dehulled rapeseeds of *Brassica napus* (cv. #10 Chinese-double) were obtained from the Institute of Oil Crops Research of the Chinese Academy of Agriculture Sciences (Wuhan, China). Alcalase 2.4L (EC 3.4.21.62, *Bacillus licheniformis*) and Pectinase (Pectinex Ultra SP-L,EC 3.2.1.15, *Aspergillus niger*) were purchased from Novo-Nodisk A/S (Bagsvaerd, Denmark). Cellulase (Cellulase AE80,EC 3.2.1.4, *Trichoderma reesei*), β-Glucanase (β-Glucanase NCB-100, EC 3.2.1.6, *Trichoderma reesei*), and Xylanase (Xylanase NCB-X50, EC 3.2.1.8, *Bacillus subtilis*) were provided by the New Century Biochemical Co. (Yueyang, China).

Carbohydrase Treatment

We first designed a short process (indicated by dotted lines in Fig. 1) to select suitable carbohydrases for subsequent hydrolysis process parameters. As such, the dehulled rapeseeds were first boiled for 5 min (seeds-to-water ratio of 1:3, w/v) to inactivate the native myrosinase, followed by wet-milling for 3 min in a mill to obtain a uniform slurry. A fixed amount of slurry (containing 100 g original dry rapeseeds) was transferred to a 1-L jacketed glass reactor connected to a thermostatically controlled water heater. The seeds-to-water ratio was fixed at 1:5 (w/v), and the slurry pH was adjusted to 5. The enzyme(s), either singly or in combination (Table 1), were then added to the slurry to a final proportion of 2.5% of the rapeseeds' weight (v/w) to commence the hydrolysis at 48°C for 3 h with an agitation rate of 200 rpm. Following the incubation, the suspension was centrifuged at 1819 *g* (3000 rpm) for 15 min. The supernatant was decanted while the precipitate was weighed, mixed, sampled for moisture content determination, and freeze-dried. The freeze-dried meal was pulverized and analyzed for residual oil. The emulsified oil yield was calculated according to the relationship expressed in Eq. 1, and a suitable enzyme or combination was selected based on high oil recovery. The enzyme combination selected, pectinase-cellulase-β-glucanase (PCG) at a ratio of 4:1:1 (v/v/v), gave the highest oil recovery (Table 1) and was subsequently applied in the single-factor optimization process. During the single-factor optimization, the effects of the seeds-to-water ratio (1:3–1:8), enzyme concentration (0–5%, v/w), and incubation time (1–7 h) were separately studied.

Optimization of Alkaline Extraction and Protease Treatment by RSM

Once the optimum conditions for the carbohydrase treatment were established, the rapeseed slurry was treated as above using PCG (4:1:1, v/v/v) at enzyme

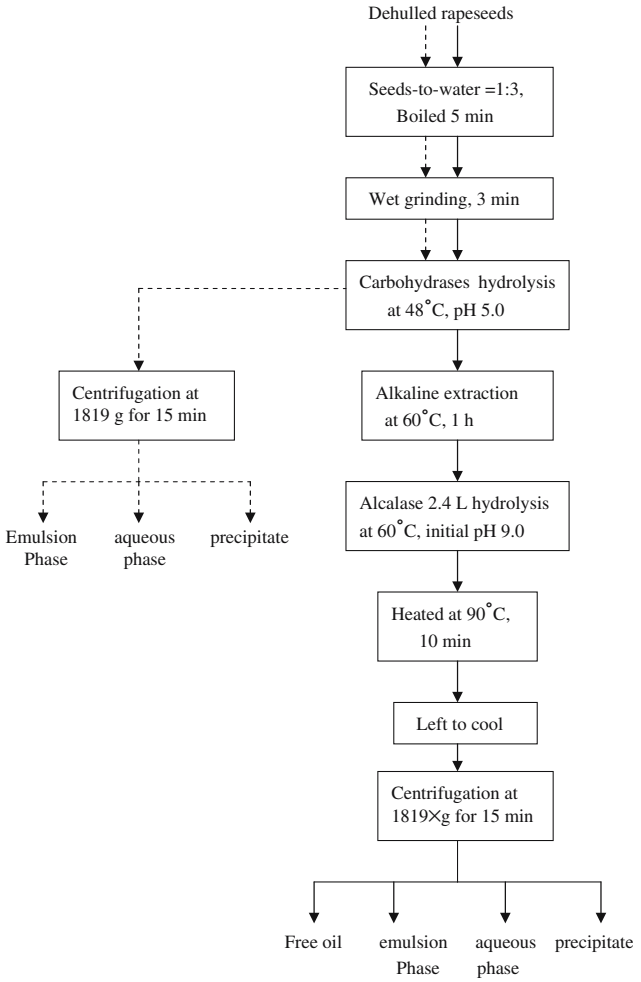

Fig. 1 The flow chart for extraction of rapeseed free oil and protein hydrolysates

Table 1 Effect of various carbohydrases on emulsified oil yield

Enzyme	Emulsified oil yield (%)
Control (without enzyme)	47.8 ± 1.2[a]
Pectinase	85.9 ± 1.2[b,c]
Cellulase	69.3 ± 1.6
β-Glucanase	64.0 ± 1.3
Xylanase	46.6 ± 2.1[a]
Pectinase + Cellulase (2:1)	88.9 ± 1.1[c,d,e]
Pectinase + Cellulase (1:1)	88.5 ± 1.0[c,d,e]
Pectinase + Cellulase (1:2)	87.0 ± 1.1[b,c,d]
Pectinase + β-Glucanase (2:1)	89.3 ± 1.7[d,e]
Pectinase + β-Glucanase (1:1)	87.2 ± 0.6[c,d]
Pectinase + β-Glucanase (1:2)	80.8 ± 2.0
Cellulase + β-Glucanase (1:1)	75.1 ± 1.4
Pectinase + Cellulase + β-Glucanase (PCG, 1:1:1)	83.9 ± 1.1[b]
PCG (3:2:1)	89.7 ± 1.8[d,e]
PCG (4:1:1)	91.6 ± 1.7 [e]

All values represent the mean of duplicate determinations ± standard deviation. Means followed by the same letter(s) are not significantly different ($P > 0.05$)

combination concentration of 2.5% (v/w) for 4 h (pH 5.0, 48°C). The subsequent alkaline extraction and protease treatment stage was optimized by RSM according to the Box-Benken design [12]. The combined effects of three independent parameters, alkaline extraction pH, Alcalase 2.4L concentration, and hydrolysis time, at three different levels each were evaluated. The parameters and levels chosen were based on the results of the preliminary experiments. To monitor the protease treatment, three responses were determined: the free oil yield from the end of the process (Y_1), the protein hydrolysates yield (Y_2), and the degree of protein hydrolysis (DH, Y_3).

The actual and coded levels of the independent variables used in the experimental design and the results obtained are shown in Table 2. The experimental plan was designed and the results statistically analyzed using DESIGN-EXPERT ver. 6.0.10 (Stat-Ease, Minneapolis, Minn.) software to build and evaluate models.

Extraction Yield

The yields of emulsified oil, free oil, and protein hydrolysates were expressed using Eqs. 1, 2, and 3, respectively.

$$\text{Emulsified oil yield, \%} = \frac{[\text{total oil in rapeseed}] - [\text{residual oil in meal}]}{[\text{total oil in rapeseed}]} \times 100\% \quad (1)$$

$$\text{Free oil yield, \% } (Y_1) = \frac{[\text{free oil}]}{[\text{total oil in rapeseed}]} \times 100\% \quad (2)$$

$$\text{Protein hydrolysates yield, \% } (Y_2) = \frac{[\text{protein in aqueous phase}]}{[\text{total protein (in rapeseed + enzyme)}]} \times 100\% \quad (3)$$

Degree of Protein Hydrolysis

The DH, defined as the percentage of peptide bonds cleaved [13], was calculated by determining the free amino groups using the ninhydrin reaction according to Doi et al. [14] with slight modifications. The original amount of free amino groups of rapeseed protein before hydrolysis was determined using a sample

of the rapeseed protein isolate. The total concentration of amino groups of rapeseed protein was determined in a sample following hydrolysis at 110°C for 24 h in 6 *N* HCl (20 mg rapeseed protein isolate in 8 mL HCl). This was regarded as 100% hydrolysis (see equation 4).

$$\text{DH}\ (\%)\ (Y_3) = \frac{[\text{free amino groups after hydrolysis}] - [\text{free amino groups before hydrolysis}]}{[\text{total amino groups of rapeseed protein}] - [\text{free amino groups before hydrolysis}]} \times 100\% \qquad (4)$$

Alkaline Extraction and Protease Treatment

This process (including the carbohydrase treatment at optimal conditions) is outlined in Fig. 1 (bold lines). Before the addition of the protease, alkaline extraction was carried out at various pH levels (9.0, 10.0, and 11.0) at 60°C for 30 min at an agitation rate of 200 rpm. At the end of each alkaline extraction condition, the pH was readjusted to 9.0 (suitable for hydrolysis with Alcalase 2.4L), while the agitation rate was decreased to 50 rpm to commence treatment with Alcalas 2.4L (0.5, 1.0, and 1.5%, v/w). The enzyme hydrolysis process was continued for various lengths of time (60, 120 and 180 min). At the end of the protease hydrolysis process, the enzyme activity was stopped by heating the reactants at 90°C for 10 min, followed by cooling to room temperature and centrifugation of the suspension at 1819 *g* for 15 min to separate the solid and liquid phases. An auto-pipettor was used to carefully draw off the free oil accumulated above the liquid phase and transfer it into a previously weighed beaker. Directly below the oil layer was a thin emulsion layer. To remove additional traces of oil from this emulsion, both the residual oil and the emulsion were transferred into a microcentrifuge tube and centrifuged as described above. The oil collected from both centrifugations was pooled and dried at 70°C in a vacuum oven to a constant weight; this dried oil was taken as the free oil recovered. The aqueous phase was collected, and its volume determined and subsequently sampled for determination of the protein content and the DH.

Molecular Weight Determination

Protein hydrolysates were submitted for molecular weight (MW) analysis using a Waters 600E Advanced Protein Purification System (Waters Corp, Milford, Mass.). A TSK gel 20005μxL (6.5 × 300 mm) column was used with 10% Acetonitrile + 0.1% trifluoroacetic acid (TFA) in high-performance liquid chromatography (HPLC)-grade water as the mobile phase. The calibration curve was obtained by running bovine carbonic anhydrase (29,000 Da), horse heart cytochrome C (12,400 Da), bovine insulin (5800 Da), bacitracin (1450 Da), gly-gly-tyr-arg (451 Da), and gly-gly-gly (189 Da). The results were obtained and processed with the aid of MILLENNIUM[32] ver. 3.05 software (Waters Corp.).

Analytical Methods

The oil contents of the initial rapeseeds and meals obtained from the short process (Fig. 1, dotted lines) were determined by the Soxhlet extraction method [15]. Protein contents of the initial rapeseeds and aqueous phases obtained from the optimized process (Fig. 1, bold lines) were determined by the Kjeldahl method (N × 6.25) [15]. All experiments were done in duplicate, and mean values of the data are reported. Statistical analysis was done using SPSS ver. 13.0 for Windows (SPSS Institute, Cary, N.C.).

Results and Discussion

Immersion of the intact rapeseeds in boiling water for 5 min was effective in destroying myrosinase, as indicated by a comparison of the amount of liberated isothiocyanate plus oxazolidine-2-thione between treated and untreated samples (results not shown), thereby resulting in an oil with lower sulfur content. The effects of the different carbohydrases on the extraction yield of the emulsified rapeseed oil are shown in Table 1. Among single enzymes used, pectinase gave a significantly ($P < 0.05$) higher oil yield (85.9%), which conforms to the fact that pectic substances are the prevalent cell-wall polysaccharides in rapeseed [4]. Additionally, the Pectinase used (Pectinex Ultra SP-L) contains partial cellulase and hemicellulase activities which probably contributed to the high oil yield. Cellulase and β-Glucanase increased the oil yield by about 44 and 34%, respectively, compared with the control, while the difference between the Xylanase treatment and the control with respect to yield was insignificant ($P > 0.05$); Xylanase was therefore not tested in subsequent experiments.

The enzymes were combined (Table 1) to evaluate their cooperative effects on extracting the oil. The

Table 2 Experimental design[a] and results obtained from the process

Run	Coded variable			Actual variable			Y_1(%)	Y_2(%)	Y_3(%)
	X_1	X_2	X_3	x_1	x_2	x_3			
1	–1	–1	0	9.0	0.5	120	66.4	70.3	16.5
2	+1	–1	0	11.0	0.5	120	66.2	75.8	15.9
3	–1	+1	0	9.0	1.5	120	71.9	77.2	19.4
4	+1	+1	0	11.0	1.5	120	71.9	79.9	19.3
5	–1	0	–1	9.0	1.0	60	69.3	68.2	17.5
6	+1	0	–1	11.0	1.0	60	68.7	74.4	18.6
7	–1	0	+1	9.0	1.0	180	73.6	77.0	20.8
8	+1	0	+1	11.0	1.0	180	74.1	78.0	20.0
9	0	–1	–1	10.0	0.5	60	66.1	75.4	16.0
10	0	+1	–1	10.0	1.5	60	73.3	76.6	18.3
11	0	–1	+1	10.0	0.5	180	70.5	74.4	17.9
12	0	+1	+1	10.0	1.5	180	75.7	83.3	21.1
13	0	0	0	10.0	1.0	120	73.5	76.3	19.3
14	0	0	0	10.0	1.0	120	71.7	78.5	17.6
15	0	0	0	10.0	1.0	120	69.9	76.9	18.0
16	0	0	0	10.0	1.0	120	72.0	77.5	18.5
17	0	0	0	10.0	1.0	120	71.0	77.2	18.4

[a] Values represent the means of two experiments. X_1, X_2, and X_3 represent the coded variables for alkaline extraction pH, Alcalase concentration, and incubation time (min), respectively; x_1, x_2, and x_3 represent the actual variables for alkaline extraction pH, Alcalase concentration (v/w), and incubation time (min), respectively; Y_1 ,Y_2, and Y_3 represent the yields of free oil and protein hydrolysates, respectively, and the degree of protein hydrolysis. Y_1 ,Y_2, and Y_3 were calculated using Eqs. 2, 3, and 4, respectively

results indicated that the top five combinations of Pectinase gave good oil recovery (>88%) but that the yields were not statistically different ($P > 0.05$). However, because we desired the highest possible oil yield (>90%), we chose the combination of PCG (4:1:1, v/v/v) for the subsequent experiments. A good recovery of emulsified oil indicates that the rapeseed cell wall was more effectively degraded by the enzyme combination, leading to the release of most of the oil and other materials enmeshed within the cells into the aqueous medium.

The seeds-to-water ratio significantly affected the extraction yield of the emulsified oil (Fig. 2). This may be due to the fact that thick suspensions prevent the effective penetration of the enzymes, while the chance of an interaction between the enzyme and substrate molecules is low in very dilute suspensions (1:7 and 1:8). The ratio of 1:5 gave the highest yield of oil, which was employed in subsequent experiments.

As is shown in Fig. 3, even a low concentration (0.2%) of PCG (4:1:1, v/v/v) had a marked effect on the extractability of oil, which was only slightly improved by higher concentrations of enzyme (more than 2.5%, v/w). However, the enzyme concentration should be a compromise between the improvement of oil recovery and the cost of enzyme. In this study, a 2.5% concentration (v/w) of PCG (4:1:1, v/v/v) was adopted.

Figure 4 shows that oil extractability was markedly affected by hydrolysis time during the first 3 h, thereafter reaching a plateau. An increase in incubation time up to 7 h did not provide any significantly higher oil yield compared with 4 h, which may be due to the depletion of the substrates and/or product inhibition of enzymes. Consequently, a period of 4 h was chosen for the PCG (4:1:1, v/v/v) treatment. When PCG at 4:1:1 (v/v/v) was used for 4 h at a concentration of 2.5%, the emulsified oil yield was determined to be 92.7% (seeds-to-water ratio: 1:5). However, no free oil was obtained, as the oil was entirely in emulsified form. This may be because (1) seed oil bodies existing in cells are surrounded by abundant proteins which prevent interaction and possible coalescence [5], and (2) the phospholipids and the protein may form lipoproteic membranes that surrounded the oil drops during grinding [4]. The protein content was determined to be about 5% in the emulsion that was formed. Thus, demulsification by hydrolyzing the protein may be a viable option. However, the emulsifying capacity (EC) of some proteins (bovine muscle and beef heart protein) increases during proteolysis until a maximum is achieved, after which it decreases with prolonged hydrolysis time [16, 17]. We therefore investigated the relationship between emulsion stability and DH by a single-factor experiment. Figure 5 shows that at a low DH (≤10%), no free oil was released and the emulsified oil extractability slightly increased compared with the control (without the addition of Alcalase 2.4L), which implies that limited hydrolysis of the protein

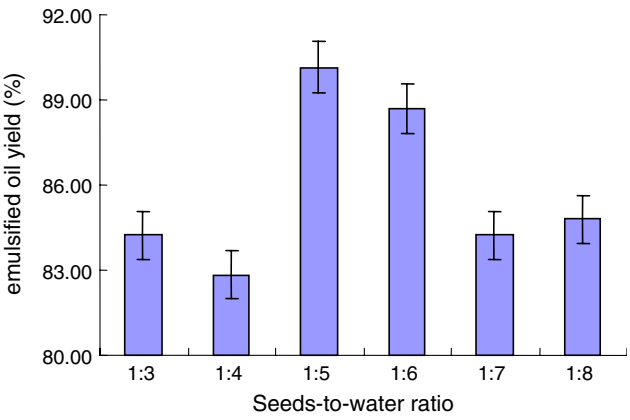

Fig. 2 Effect of seeds-to-water ratio on emulsified oil yield following extraction with a 2.5% concentration (v/w) of a combination of pectinase, cellulase, and β-glucanase (PCG; 4:1:1, v/v/v) for 3 h with gentle agitation

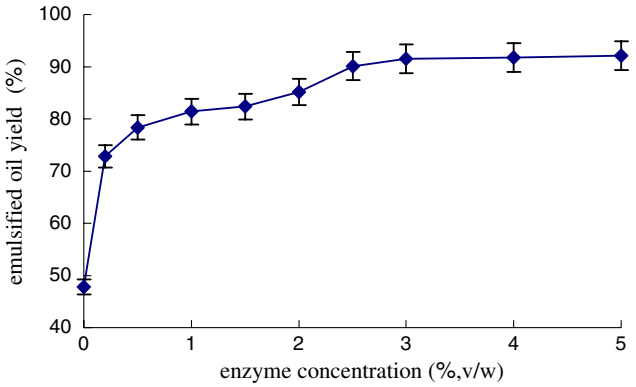

Fig. 3 Effect of the concentration of the combination of PCG (4:1:1, v/v/v) on emulsified oil yield. Conditions: seeds-to-water ratio, 1:5; 3-h incubation; gentle agitation

may improve its EC. However, as the DH rose above 10%, notable amounts of free oil could be obtained, which then increased remarkably between 10 and 12% DH. This latter result indicates that once the protein is extensively hydrolyzed the emulsion will become unstable and the emulsified oil can be released.

Following the carbohydrase treatment, alkaline extraction was carried out to solubilize more protein in the aqueous phase and hence enhance the protease hydrolysis and protein extractability. Table 2 shows the experimental conditions and the results obtained from the RSM optimization. The values of Y_1 (free oil yield), Y_2 (protein hydrolysates yield), and Y_3 (DH) ranged from 66.1 to 75.7, from 68.2 to 83.3, and from 15.9 to 21.1%, respectively. The maximum values of the three responses were at the same experimental conditions, i.e., an alkaline extraction pH of 10.0, an Alcalase 2.4L concentration of 1.5% (v/w), and a hydrolysis time of 180 min. The minimum values of Y_1, Y_2, and Y_3 were at pH 10.0, 0.5%, and 60 min, pH 9.0, 1.0%, and 60 min, and pH 11.0, 0.5%, and 120 min, respectively.

The data were analyzed employing a multiple regression technique to develop response surface models. Both a linear model and a second-order model were tested, using *an F*-test at the 95% confidence level. The following three second-order models satisfactorily explained the free oil yield, the protein hydrolysates yield, and the DH with non-significant lack of fit (Table 3). Y_{oil}, Y_{protein}, and Y_{DH} are the predicted values for free oil yield (%), protein hydrolysates yield (%), and DH (%), respectively, and X_1, X_2, and X_3 are the coded variables as described in Table 3.

$$Y_{\text{oil}}\ (\%) = 71.61 - 0.046X_1 + 2.95X_2 + 2.05X_3 - 1.22X_1^2 - 1.30X_2^2 + 1.07X_3^2 + 0.057X_1X_2 + 0.28X_1X_3 - 0.51X_2X_3 \quad (5)$$

$$Y\text{protein}\ (\%) = 77.29 + 1.91X_1 + 2.64X_2 + 2.26X_3 - 2.25X_1^2 + 0.77X_2^2 - 0.63X_3^2 - 0.73X_1X_2 - 1.30X_1X_3 + 1.91\ X_2X_3 \quad (6)$$

$$Y_{\text{DH}}\ (\%) = 18.35 - 0.060X_1 + 1.48X_2 + 1.16X_3 + 0.15X_1^2 - 0.71X_2^2 + 0.70X_3^2 + 0.10X_1X_2 - 0.46X_1X_3 + 0.21X_2X_3 \quad (7)$$

On the basis of Fig. 6a and Eq. 5, which shows variable X_2 and variable X_3 with large regression coefficients (positive), it can be concluded that by increasing both the Alcalase 2.4L concentration and hydrolysis time, we significantly increased the free oil

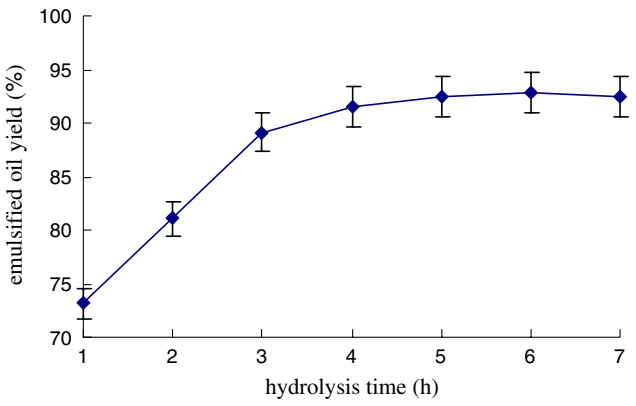

Fig. 4 Effect of varying hydrolysis time on emulsified oil yield. Conditions: seeds-to-water ratio,1:5; 2.5% concentration (v/w) of the combination of PCG (4:1:1, v/v/v); gentle agitation

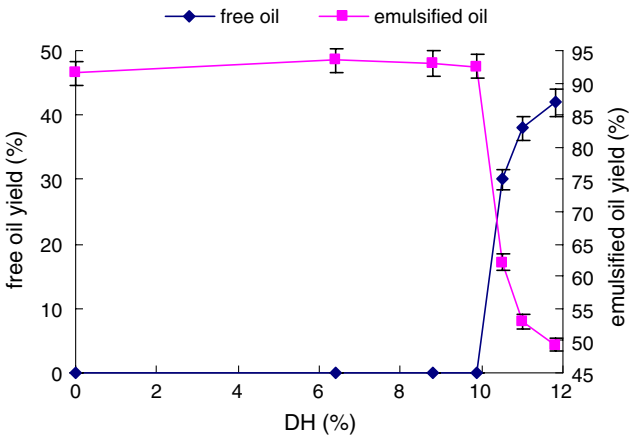

Fig. 5 Effects of degree of protein hydrolysis (*DH*) on the yields of free oil and emulsified oil

yield. We also observed that the two variables had similar effects on yield of the protein hydrolysates and DH (Fig. 6b; Eqs. 6 and 7), respectively. Figure 6c shows that increasing the pH values led to a concomitant increase in the protein yield at low enzyme concentrations, while a higher yield was not obtained at the highest values of both pH (11.0) and enzyme concentration (1.5%). This implies that the yield in protein hydrolysates depended more on the pH of the alkaline extraction when the extraction was followed by protein hydrolysis at a lower DH. Rapeseed protein has been reported to be more extractable at a higher pH [18, 19]. On the other hand, because protease can hydrolyze and extract the non-soluble protein, if a higher DH was reached in the following step, the alkaline extraction pH may not be the predominant factor that significantly affected the yield of the protein hydrolysates. Conversely, the alkaline extraction pH had no significant effect on the free oil yield and DH (figures not shown). The different effects of the pH of the alkaline extraction on the three responses can also be confirmed by a comparison of the regression coefficients of the variable X_1 in the three equations (Eqs. 5, 6, and 7) [20]. The variable X_1 had the larger regression coefficient for Y_{protein} (+1.91) than those for Y_{oil} and Y_{DH} (–0.046 and –0.060, respectively). The results indicate that a higher free oil yield is accompanied by a higher DH and protein hydrolysates yield. It can be deduced that more oil is liberated when the protein is hydrolyzed to a higher DH.

The increase in the yields of free oil extraction and the protein hydrolysates are the two factors that are the most objective indicators of the efficiency of the aqueous enzymatic process. Therefore, the optimization of the treatment should be based on both. On the basis of the above discussion, the best conditions for the oil and protein extractions were found to be a high concentration (>1.25%) of Alcalase 2.4L and a long hydrolysis time (>150 min). The alkaline extraction pH showed a significant effect on the protein hydrolysates yield, but not on the free oil yield. When the extraction pH was below 10 and the protease was used at a low concentration (<1%), the yield of protein hydrolysates decreased drastically. As shown in Fig. 6a and b, when the alkaline extraction pH was fixed at 10 and the concentration of Alcalase 2.4L ranged from 1.25 to 1.5% during a 150- to 180-min hydrolysis time, the free oil and the protein hydrolysates yields were 73–76% and 80–83%, respectively.

Protein hydrolysates rich in small peptides were obtained by the optimized aqueous enzymatic extraction process using a combination of carbohydrases followed by Alcalase 2.4L protease extraction after a subsequent alkaline pH extraction. According to the high-performance size-exclusion chromatography (HP-SEC) results, the hydrolysates consisted of approximately 96% peptides that had a MW less than 1500, of which about 81% had MW less than 600 Da. These small peptides have a wide range of potential applications for the food, cosmetics, and/or pharma-

Table 3 Analysis of variance for response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	*F* value	$P > F^a$
Free oil yield (%)					
Model	122.17	9	13.57	11.05	0.0023
Residual	8.60	7	1.23		
Lack of fit	1.32	3	0.44	0.24	0.8628
Pure error	7.27	4	1.82		
Total	130.77	16			
Coefficient of variation = 1.56%, R^2 = 0.9342					
Protein hydrolysates yield (%)					
Model	174.79	9	19.42	15.67	0.0008
Residual	8.67	7	1.24		
Lack of fit	6.00	3	2.00	3.00	0.1584
Pure error	2.67	4	0.67		
Total	183.46	16			
Coefficient of variation = 1.46%, R^2 = 0.9527					
DH (%)					
Model	33.40	9	3.71	13.89	0.0011
Residual	1.87	7	0.27		
Lack of fit	0.18	3	0.061	0.14	0.9282
Pure error	1.69	4	0.42		
Total	35.27	16			
Coefficient of variation = 2.81%, R^2 = 0.9470					

[a] $P < 0.05$ indicates statistical significance

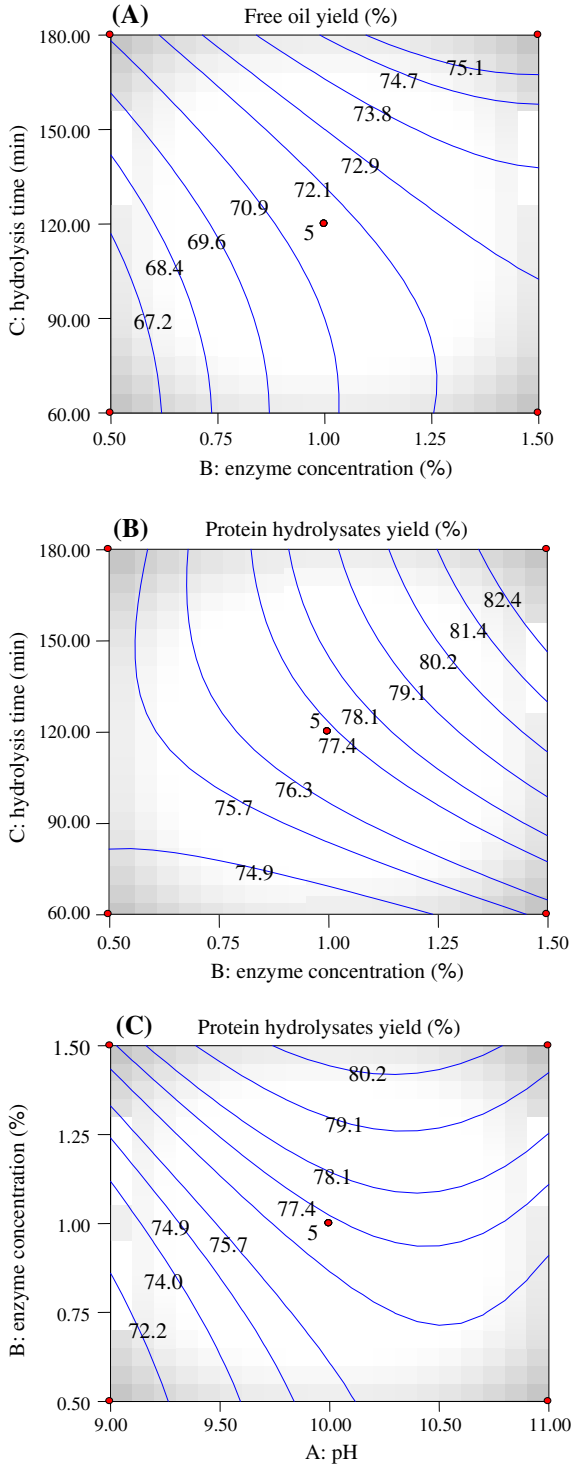

Fig. 6 Response contours for the yield of free oil (**a**) and protein hydrolysates (**b**) as a function of Alcalase 2.4L concentration and hydrolysis time at pH 10. **c** Yield of protein hydrolysates as a function of alkaline extraction pH and Alcalase 2.4L concentration during a 120-min hydrolysis

ceutical industries [21]. Our results show the important effect of Alcalase 2.4L; i.e., appreciable amounts of the free oil and protein hydrolysates were simultaneously obtained. Thus, effective demulsification and potential applications of protein hydrolysates should result in this environmentally friendly process becoming a viable technology for rapeseed processing in the future.

References

1. FAO/WHO/UNU (2002) Agricultural bulletin board on data collection, dissemination and quality of statistics. World Health Organization, Geneva
2. Henryk N (1990) Rapeseed. Elsevier, Amsterdam
3. Marlowe IT, Giddings TJ, Richardson SJ, Stentiford A (1991) UK industry and ozone pollution from volatile organic compound emissions. Warres Spring Laboratory, Report 878. The Environmental Technology Executive Agency of the Department of Trade and Industry, London
4. Domínguez H, Nunez MJ, Lema JM (1994) Enzymatic pretreatment to enhance oil extraction from fruits and oilseeds: a review. Food Chem 49:271–286
5. Rosenthal A, Pyle DL, Niranjan K (1996) Aqueous and enzymatic processes for edible oil extraction. Enzyme Microb Technol 19:402–420
6. Cater CM, Rhee KC, Hagenmaier RD, Mattil KF (1974) Aqueous extraction—an alternative oilseed milling process. J Am Oil Chem Soc 51:137–141
7. Olsen HAS (1981) Method of producing soy protein hydrolysate from fat-containing soy material, and soy protein hydrolysate. U.S. Patent 4,324,805, Washington D.C.
8. Yust MM, Pedroche J, Megias C, Calle JG, Alaiz M, Millan F, Vioque J (2004) Rapeseed protein hydrolysates: a source of hiv protease peptide inhibitors. Food Chem 87:387–392
9. Lanzani A, Petrini MC, Cozzoli O, Gallavresi P, Carola C, Jacini G (1975) On the use of enzymes for vegetable-oil extraction. A preliminary report. Riv Ital Sostanze Grasse L11:226–229
10. Fullbrook PD (1983) The use of enzymes in the processing of oilseeds. J Am Oil Chem Soc 60:476–478
11. Jensen SK, Olsen HS, Sørensen H (1990) Aqueous enzymatic processing of rapeseed for production of high quality products. In: Shahidi F (ed) Canola and rapeseed: production, chemistry, nutrition and processing technology. Van Nostrand Reinhold, New York, pp 331–344
12. Myers RH, Montgomery DC (1995) Response surface methodology: process and product optimization using designed experiments. John Wiley & Sons, Chichester
13. Jens AN (1986) Enzymatic hydrolysis of food proteins. Elsevier, London
14. Doi E, Shibata D, Matoba T (1981) Modified colorimetric ninhydrin method for peptidase assay. Anal Biochem 118:173–184
15. Association of official analytical chemists (AOAC) (1995). Official methods of analysis, 16th edn. AOAC International, Gaithersburg
16. Dubois MW, Anglemier AF, Montgomery MW, Davidson WD (1972) Effect of proteolysis on the emulsification characteristics of bovine skeletal muscle. J Food Sci 37:27–28
17. Smith DM, Brekke CJ (1984) Functional properties of enzymatically modified beef heart protein. J Food Sci 49:1525–1528
18. Xu L, Diosady LL (1994) The production of Chinese rapeseed protein isolates by membrane processing. J Am Oil Chem Soc 71:935–939

19. Klockeman DM, Toledo R, Sims KA (1997) Isolation and characterization of defatted canola meal protein. J Agric Food Chem 45:3867–3870
20. Cheison SC, Wang Z, Xu SY (2006) Hydrolysis of whey protein isolate in a tangential flow filter membrane reactor-I: characterisation of permeate flux and product recovery by use of multivariate data analysis. J Membrane Sci 283:45–56
21. Tessier B, Schweizer M, Fournier F, Framboisier X, Chevalot I, Vanderesse R, Harscoat C, Marc I (2005) Prediction of the amino acid composition of small peptides contained in a plant protein hydrolysate by LC-MS and CE-MS. Food Res Int 38:577–584