Technological optimizatation for hydrolysis of rapeseed albumin with alcalase

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Abstract: The limited hydrolysis process for rapeseed albumin (RSA) with alcalase was systematically studied through response surface methodology (RSM). The optimum conditions were established, which included hydrolyzing temperature (50℃), enzyme concentration (0.38 Anson Units per gram of substrate) and concentration of substrate (4.87%). The gel filtration chromatography (Sephadex G-25) profile showed the major albumin protein was degraded after hydrolysis. In addition, the amino acid profiles indicated that hydrolyzed rapeseed albumin could be used as an additive with great potential in food industry.

Key words: defatted rapeseed meal; enzymatic hydrolysis; rapeseed albumin; alcalase; degree of hydrolysis; response surface methodology (RSM)

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1 Introduction

Rapeseed, one of the most important oilseed crops cultivated in the world is becoming of increasing interest as a source of edible proteins. Rapeseeds contain 35%～47% of protein, and hence defatted rapeseed meal may constitute a good source of proteins for humans. Its amino acid composition is well-balanced in regard to FAO requirements. Moreover, oilseed protein is rich in sulfur-containing amino acids and lysine which are generally limited in legumes and cereals[1,2].

Protein function can be modified by enzymatic hydrolysis, which alters, for instance, solubility, viscosity, emulsion and foam properties[3]. It has been reported that enzymatic protein hydrolysates could be served as suitable sources of protein for human nutrition because of their gastrointestinal absorption, especially di- and tripeptides which seem to be more effective than both intact protein and free amino acid[4]. Therefore, in order to improve nutritional and functional properties of protein, protein hydrolysates have been widely used in specific formulation.

Alcalase is a microbial protease from the bacterium *Bacillus licheniform is* with endopeptidase activity, which can hydrolyze protein into short peptides. Response surface methodology (RSM) was originally described by Box and Wilson[5] as being effective for responses that were influenced by many factors and their interactions.

In the present paper, rapeseed albumin (RSA) was used as starting material for the generation of rapeseed peptide (RSP). The hydrolysis was carried out using an endopeptidase (alcalase) with a function to produce an limited hydrolysate. RSM was designed to optimize the hydrolysis conditions. It was also discussed the amino acid profiles of albumin and hydrolyzed rapeseed albumin. The results and data could provide a theoretical basis for extensive application of hydrolyzed rapeseed albumin in food industry.

2 Materials and methods

2.1 Materials

Rapeseed albumin (RSA): They are self-refined from undulled and defatted rapeseed meal (81% crude protein, calculated as N %×6.25).

Proteolytic enzyme: The enzyme is alcalase 2.4 L (Novo Nordisk, Bagsvaerd, Denmark), a protease of *Bacillus licheniform is* with endopeptidase activity. The main component of the commercial preparation is serine protease subtilisin A. The specific activity of alcalase 2.4 L is 2.4 Anson Units (AU) per gram. One AU is the amount of enzyme that, under standard conditions (pH 8.0), digests hemoglobin at an initial rate that produces an amount of trichloroacetic acid-soluble product that gives the same color with the Folin reagent as 1meq of tyrosine released per minute.

All chemicals including Blue Dextran 2000 (Pharnacial), Bactiracin (Sigma), Glutathione

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(Reduced, Amresco) were of analytical grade.

2.2 Materials and methods

2.2.1 Heat treatment

Heat-treatment has been reported as an effective way to improve the degree of hydrolysis protein[6]. Therefore, heat denaturing of the RSA was pretreated at 80℃ for 30 min using a water bath. RSA was mixed intermittently to ensure well-distributed heating during the process of heat treatment.

2.2.2 Total nitrogen determination

Total nitrogen was determined according to the micro-Kheldahl method[7,8]. Crude protein content was calculated using a conversion factor of 6.25.

2.2.3 Enzymatic hydrolysis of rapeseed albumin

The method of Javier Vioque et al[3] was used for enzymatic hydrolysis of rapeseed albumin. The RSA of assumed concentration was hydrolyzed batchwise in a vessel equipped with a stirrer, a thermometer, and a pH-electrode. Hydrolysis was carried out for 60 min using the assumed hydrolysis parameters including temperature (T), enzyme/substrate ratio (E/S) and substrate concentration (S). Hydrolysis was terminated by heating at 90℃ for 8 min. Hydrolysates were clarified by filtration to remove insoluble substrate fragments. The filtrate was lyophilized after isolation each time and kept freeze-dried for next use.

2.2.4 Measurement of degree of hydrolysis

The degree of hydrolysis (DH), defined as the ratio of amino nitrogen/total nitrogen (AN/TN), was calculated according to the method of Zhao Xinhua et al[9,10].

$$DH\% = AN\ (\text{amino nitrogen}) / TN\ (\text{total nitrogen}) \times 100\%$$

The AN, produced by hydrolyzing, was determined by formaldehyde titration procedure. Total nitrogen was determined according to the micro-Kheldahl method mentioned above (2.2.2).

2.2.5 Optimization of hydrolytic conditions

A three-factor central composite design was employed to examine the response, degree of hydrolysis ($DH\%$) of RSA by alcalase as changed with the independent variables, temperature (℃, X_1), concentration of enzyme (AU/g protein, X_2) and concentration of substrate (%, X_3). A quadratic polynomial regression model was assumed for predicting the response. Every factor (Code X_1 to X_3) had three levels corresponding to three code values. There were totally 15 independent experiments. In every experiment, levels of the factors were arranged according to table 1. The model proposed was described in table 2. Experimental data were analyzed for response surface regression for a quadratic polynomial model using SAS software (SAS Institute Inc. 1990).

Table 1 Design of factors and levels in experiments

Factor	Code	Code value	Level
T/℃	X_1	+1	45
		0	50
		-1	55
E/S/AU·g^{-1}	X_2	+1	0.2
		0	0.3
		-1	0.4
S/%	X_3	+1	3
		0	5
		-1	7

Table 2 Different levels of factors arranged in experiments

Test number	Code value of experiment		
	X_1	X_2	X_3
1	-1	-1	0
2	-1	0	-1
3	-1	0	+1
4	-1	1	0
5	0	-1	-1
6	0	-1	+1
7	0	+1	-1
8	0	+1	+1
9	+1	-1	0
10	+1	0	-1
11	+1	0	+1
12	+1	+1	0
13	0	0	0
14	0	0	0
15	0	0	0

2.2.6 Gel filtration chromatography

Gel filtration was carried out in system equipped with a Sephadex G-25 column (50 cm × 2.6 cm) and distilled water as eluent at a flow rate of 0.5 mL/min. Elution was monitored at 280 nm and the fractions were collected every 10 min intervals. The molecular masses were determined using a calibration curve made with Blue Dextran 2000 (2000 kDa), Bactiracin (Sigma, 1422 Da), and Glutathione (Reduced, Amresco, 307 Da) which were used as molecular weight standards.

2.2.7 Amino-acid analysis

Amino-acid analysis of HCl-hydrolyzed samples was carried out using an automated Beckman instrument. This work was completed by the amino-acid analysis service of the Oil Institute of the Chinese Academy of

Agricultural Sciences (CAAS). All amino acid data were corrected for 100% recovery.

3 Results and discussion

3.1 Effect of material pre-processing on hydrolysis degree

It has been documented that protein coagulates at 80℃, and shows changes in the ordered structure with increased surface hydrophobicity, decreased amount of sulphydryl groups[6]. Therefore, heat-denaturing of protein can cause the molecules to unfold and become more accessible to proteases for hydrolytic reaction than in their native state in theory. Figure 1 showed that the hydrolysis values only improved a little when RSA was treated at 80℃ for 30 min before the hydrolysis experiment. The enhanced effect on *DH* is not significant possibly because the RSA structure is simple (only 1.7S protein). Therefore the materials of the following experiments were still used without heat-treatment.

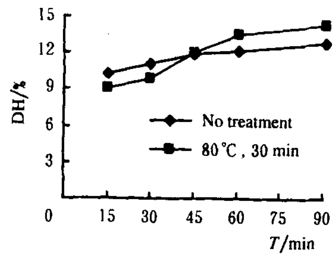

Fig. 1 Effect of heat treatment on hydrolysis of rapeseed albumin with alcalase (0.3AU/g protein)

3.2 Optimization of technology for RSA hydrolysis

Results of 15 experiments were shown in table 3. Content of hydrolysis degree (*DH*) were used as response values in analysis of response surface regression (RSREG). The equation $DH\ (Y) = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3$ was used as regression model. The procedure RSREG of SAS also gave values of parameter estimated (table 4) and predicted values of the equation (table 5).

Table 3 Degree of hydrolysis of 15 experiments

Test number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Degree of hydrolysis/%	5.37	7.11	5.58	12.89	8.39	5.79	13.39	12.92	6.09	11.06	10.25	10.43	14.25	14.23	14.29

Table 4 Parameters estimated by regression model

Parameter	a_0	a_1	a_2	a_3	a_{11}	a_{22}	a_{33}	a_{12}	a_{13}	a_{23}
Value in model about degree of hydrolysis	-407.468	14.927	214.35	3.375	-0.144	-196.958	-0.541	-1.590	0.018	2.663

Table 5 Predicted values of regression model

Response variable			Degree of hydrolysis/%	
T /℃	E/S /AU · g^{-1}	S /%	Calculated value[a]	Observed value[b]
50.07	0.38	4.87	14.61	14.72

Note: a—Calculated using the predicted equation;
b—Mean value of three replications of the hydrolysis experiment.

Table 6 Variance analysis of regression equations

Variance source	Degree of freedom	Degree of hydrolysis		
		Sum of square	Mean square	F Value
Model	9	155.259	17.251	6.39*
Error	5	13.494	2.699	
Correct total	14	168.753		
Linearly dependent coefficient		$R^2 = 0.9591$		

Note: ** $f_{0.01}(9, 5) = 10.2$; * $f_{0.05}(9, 5) = 4.8$

Variance analysis of regression equation was conducted (table 6). F value of the model was bigger than $f_{0.05}(9, 5)$. R^2 was 0.9591, which showed that linear relationship between dependent variable and whole independent variables was significantly distinct.

Figure 2 was response surface diagrams of *DH*. High concentration of enzyme (E/S) and low substrate concentration (S) were good for degree of hydrolysis. Considering the interaction of all the variables, the optimum conditions for hydrolyzing rapeseed albumin with alcalase can be calculated by the assumed equation as follows, hydrolyzing temperature: 50℃, enzyme concentration: 0.38AU per gram of substrate, concentration of substrate: 4.87%. The degree of hydrolysis of rapeseed albumin can reach 14.72% after reacting 1 h on this optimum condition. The values obtained from validation experiment showed a very good agreement with the predicted values (table 5). Consequently, the regression model could be used to analyze the results of the experiment and to predict. The model was also applied to choose the perfect hydrolysis conditions for the limited hydrolysis of rapeseed albumin.

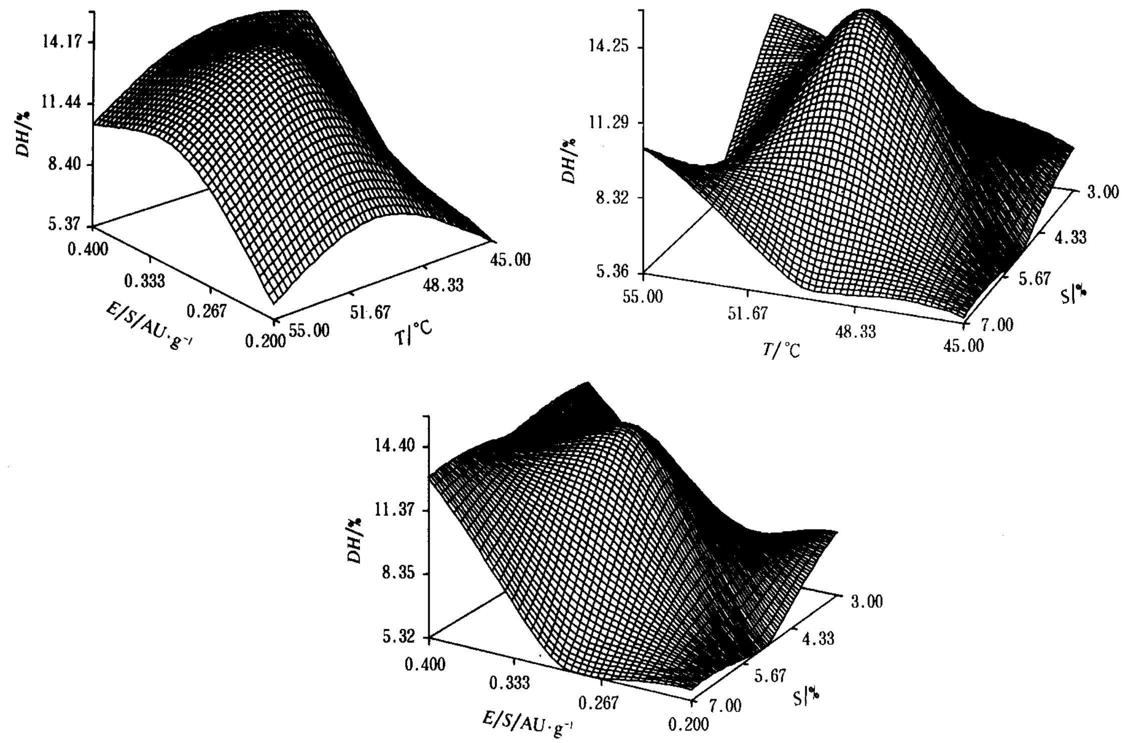

Fig. 2 Response surface diagrams of degree of hydrolysis

3.3 Gel filtration chromatography

RSA profile on gel filtration was characterized by the presence of the major protein component of protein isolated from rapeseed that corresponds to the 1.7S albumin, with molecular masses much larger than 5 kDa (Fig. 3). As a result of the hydrolysis process, RSA hydrolysates showed a major chromatographic peak of intermediate molecular mass between 5 kDa and 1 kDa. Among the final hydrolysate, the relative amount of this peak decreased while the amounts of others with lower molecular weights increased. Thus, after hydrolyzing for 60 min, the maximal protein absorbance changed. The results showed that the molecular weight of rapeseed albumin hydrolysates decreased with an increase in degree of hydrolysis.

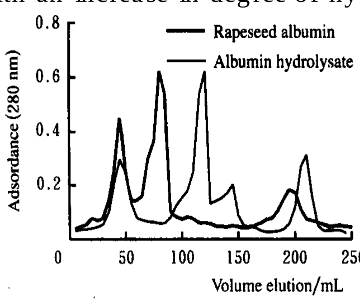

Fig. 3 Gel filtration chromatography of rapeseed albumin and protein hydrolysate after they were treated by alcalase for 60 min

3.4 Amino acid composition

The amino acid composition of RSA was similar to the amino acid composition of Zhongshuang 119 albumin[11]. Glutamic acid, leucine, aspartic acid and proline were the dominant amino acids accounting for more than 40% of the total amino acids.

Table 7 Percentage of amino acid in albumin and its hydrolysates %

Amino acid	Hua-Za 3 Albumins	Albumins hydrolysate	Ref value[11]
Essential			
Isoleucine	3.77	3.89	2.87
Leucine	8.17	7.77	11.48
Lysine	4.55	3.05	5.54
Methionine	2.54	2.79	2.73
Phenylalanine	7.61	10.70	5.15
Threonine	5.91	6.17	4.81
Valine	3.69	3.32	5.84
Tryptophan	-	-	6.64
Nonessential			
Tyrosine	3.79	7.47	2.89
Cystine	2.64	1.85	0.34
Histidine	6.94	8.92	2.80
Analine (Ala)	3.23	3.06	9.96
Arginine	8.30	5.42	-
Aspartic acid	7.28	6.27	4.15
Glutamic acid	13.97	12.53	15.56
Glycine	6.53	6.52	6.59
Proline	7.22	5.99	7.33
Serine	3.86	4.28	5.33

The amino acid composition of RSA hydrolysate and RSA were similar (table 7) except for some gained in Tyrosine, Phenylalanine and loss in Cystine, Lysine and Arginine for specificity of alcalase, a bacterial endopeptidase. The results indicat that the process of enzymatic hydrolysis is a gentle procedure that do not change the amino acid profile of the starting protein significantly. The final hydrolysate of rapeseed albumin show the high solubility and gastrointestinal absorption, which make them become the suitable materials for the supplementation of liquid foods or high-energy beverages.

The hydrolysate compared favorably with the FAO/WHO (1985)[12] suggested pattern of amino acid requirements for adults, school children, and preschool children (table 8). Compared to the standards for adults, RSA hydrolysate only has a modest deficiency in the lysine content. Therefore, RSA hydrolysate can also be used as a potential ingredient for formulas for adults.

Table 8 Comparison of FAO/WHO (1985)-suggested pattern of amino acid requirements[12] with the composition of RSA hydrolysate (mg/100 mg protein)

Amino acid	Suggested pattern of requirement				Composition of RSA hydrolysate
	Infant[a]	Preschool child[b]	School child[c]	Adult	
Histidine	2.6	1.9	1.9	1.6	3.9
Isoleucine	4.6	2.8	2.8	1.3	1.7
Leucine	9.3	6.6	4.4	1.9	3.4
Lysine	6.6	5.8	4.4	1.6	1.3
Methionine+cystine	4.2	2.5	2.2	1.7	2.0
Phenylalanine+tyrosine	7.2	6.3	2.2	1.9	7.9
Threonine	4.3	3.4	2.8	0.9	2.7
Tryptophan	1.7	1.1	0.9	0.5	-
Valine	5.5	3.5	2.5	1.3	1.5

Note: a—Averaged amino acid composition of human milk;
b—2~5 years;
c—10~12 years

4 Conclusions

Heat-treatment could not significantly improve the *DH* of rapeseed albumin digested by alcalase. The optimum conditions for hydrolyzing rapeseed albumin with alcalase were established by response surface methodology. These parameters included hydrolyzing temperature: 50℃, enzyme concentration: 0.38AU per gram of substrate, concentration of substrate: 4.87%. After the reaction was conducted for 1 h under this optimum condition, the degree of hydrolysis of rapeseed protein isolated can reach 14.72%. The results obtained from this pattern tallied with the actual experiment results. Therefore, the response surface methodology could be used to predict the *DH* of rapeseed albumin hydrolysates, and to screen the perfect hydrolysis conditions for the limited hydrolysis of rapeseed albumin. Hua-Za 3 rapeseed is very low in both erucic acid and glucosinolates. Consequently, the RSA hydrolysate with high quality is one of the ideal protein sources for human nutrition products with high-added value in the future.

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碱性蛋白酶(alcalase)水解菜籽清蛋白的工艺优化

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摘 要: 采用响应曲面法对碱性蛋白酶(alcalase)水解菜籽清蛋白工艺进行系统地研究。确定最佳水解条件如下: 温度50℃、酶浓度 0.38 AU/g、底物浓度 4.87%。同时, 葡聚糖凝胶(Sephadex G-25)柱层析显示水解物较原清蛋白分子量变小。氨基酸组成分析结果表明菜籽清蛋白水解物可作为一种营养丰富的食品添加剂加以广泛利用。
关键词: 脱脂菜籽饼粕; 酶解; 菜籽清蛋白; 碱性蛋白酶(alcalase); 水解度; 响应曲面法